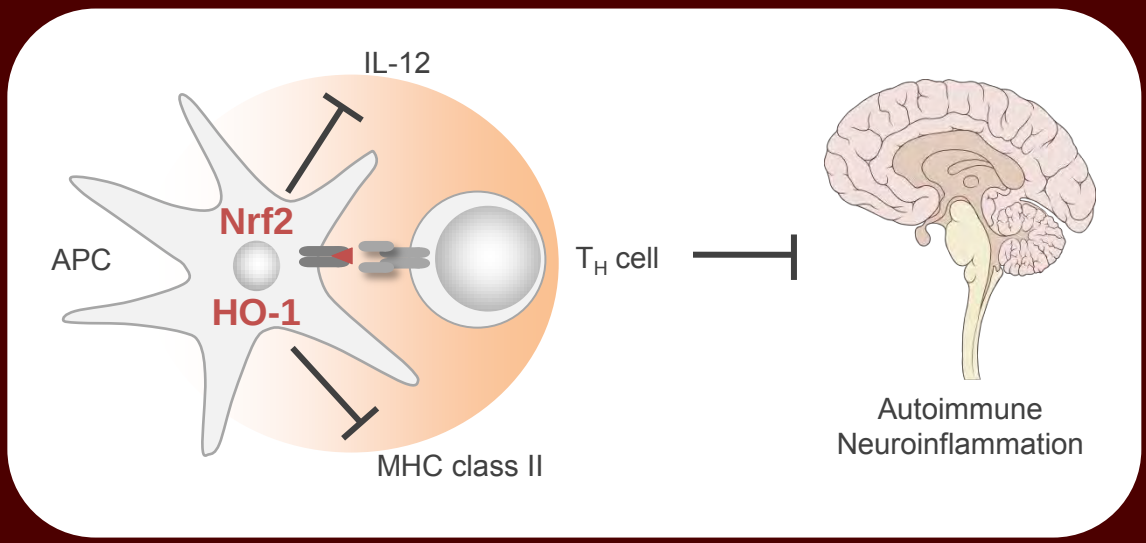
Regulation of Autoimmune Neuroinflammation by Stress-responsive Genes

Andreia Cristina Marques da Cunha



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
March, 2012

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Knowledge Creation

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Ph.D Supervisor: Dr. Miguel P. Soares

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Index

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On a very personal note a special thank you goes to my companion, my parents and friends who have been throughout the years an endless source of strength. We are who we love and this Thesis is also yours.

Abbreviations

APC - antigen-presenting cell(s)
APM - ambient particulate matter
ARE - antioxidant response element
ATF - activating transcription factor
ATP - adenosine triphosphate
BBB - blood-brain barrier
BCB - blood-CSF barrier
BTB - Broad-Complex, Tramtrack, and Bric-a-brac
bZip - basic leucine-zipper
Cat - catalase gene
CBP - CREB binding protein
Ccl20 - CC-chemokine ligand 20
Ccr6 - CC-chemokine receptor 6
CD - cluster of differentiation
CFA - complete Freund's adjuvant
CIITA - MHC class II transcription activator
CLRs - C-type lectin receptors
CNC - cap 'n' Collar
CNS - central nervous system
CO - carbon monoxide
Con A - concanavalin A
CoPPIX - cobalt protoporphyrin IX
CREB - cyclic-AMP-responsive-element-binding protein
CSF - cerebrospinal fluid
CTLA - cytotoxic T-lymphocyte antigen
Cul3 - Cullin 3
DAMPs - damage-associated molecular patterns
DC - dendritic cell(s)
EAE - experimental autoimmune encephalomyelitis
EBV - Epstein Bar virus
EC - endothelial cell(s)
ERK - extracellular signal-regulated kinase
Foxp3 - forkhead box P3
Fth/FtH - ferritin H chain gene/protein
GATA3 - trans-acting T cell-specific transcription factor 3
Gclc - glutamate-cysteine ligase, catalytic subunit gene
Gclm - glutamate-cysteine ligase, modifier subunit gene
GM-CSF - granulocyte macrophage colony-stimulating factor
Gpx - glutathione peroxidase gene
GSK - glycogen synthase kinase
Gsr - glutathione reductase gene
Gstt - glutathione *S*-transferase gene
HLA - human leukocyte antigen
HMGB1 - high-mobility group box 1 protein
Hmox1/HO-1 - heme oxygenase 1 gene/protein
IFN - interferon

Ig - immunoglobulin
IL - interleukin
IRF - interferon regulatory factor
JAK - Janus kinase
JNK - c-Jun N-terminal kinase
Keap1 - Kelch-like ECH associated protein 1
LN - lymph node(s)
LPS - lipopolysaccharide
Mø - macrophage(s)
mAb - monoclonal antibody
Maf - musculo-aponeurotic fibrosarcoma oncogene
MAPK - mitogen-activated protein kinase
MBP - myelin basic protein
MEF- mouse embryonic fibrobast(s)
MHC - major histocompatibility complex
MOG - myelin oligodendrocyte glycoprotein
MyD88 - myeloid differentiation primary response gene 88
MS - multiple sclerosis
NADPH - nicotinamide adenine dinucleotide phosphate
NF-AT - nuclear factor of activated T cells
NF-E2 - nuclear factor erythroid 2
NF-κB - nuclear factor kappa B
NGS - normal goat serum
NLRs - NOD-like receptors
NO - nitric oxide
Nrf2 - NF-E2-related factor 2
Nqo - NAD(P)H:quinone oxidoreductase gene
MAC - membrane attack complex
Mt - metallothionein gene
PAMPs - pathogen-associated molecular patterns
PBS - phosphate-buffered saline
PLP - proteolipid protein
PMA - phorbol 12-myristate 13-acetate
PP-MS - primary progressive multiple sclerosis
PPR-γ - proliferator-activated receptor-γ
Prdx - peroxiredoxin gene
PRRs - pattern-recognition receptors
PTx - *Pertussis* toxin
Rag - recombination activating gene
Rbx1 - Ring-box1 protein
RLRs - RIG I-like receptors
RNS - reactive nitrogen species
ROR - retinoic acid receptor-related orphan receptor
ROS - reactive oxygen species
RR-MS - relapsing-remitting multiple sclerosis
SP-MS - secondary progressive multiple sclerosis
STAT - signal transducer and activator of transcription
Sod - superoxide dismutase gene
T-bet - T-box transcription factor

T_C - cytotoxic T cell

TCR - T cell receptor

T_{FH} - follicular helper T cell

TGF-β - transforming growth factor-β

T_H - Helper T cell

TLRs - Toll-like receptors

TNF - tumor necrosis factor

TRAM - TRIF-related adaptor molecule

TIRAP - TIR-associated protein

T_{REG} - regulatory T cell

TRIF - TIR-domain-containing adaptor protein-inducing IFN-β

Trn - thioredoxin gene

ZnPPIX - zinc protoporphyrin IX

Preface

This Thesis describes the data obtained during the research work performed at the Instituto Gulbenkian de Ciência under the scientific supervision of Dr. Miguel P. Soares.

It is organized in 4 chapters and two appendixes, preceded by an abstract written both in Portuguese and English. A state of the art introductory review on the subject is provided in Chapter 1. In Chapters 2, 3 and Appendix 1 the original observations obtained during the research period are presented and discussed. Chapter 5 consists of a general discussion aiming at integrating the results presented in the previous chapters. Appendix 2 is a review manuscript published during this time on a relevant topic for this Thesis.

Sumário

A inflamação é uma resposta protetora iniciada por células da imunidade inata, em resposta a uma infeção ou lesão dos tecidos, cujo objetivo é eliminar a fonte de infeção ou o estímulo danoso. A resposta inflamatória desempenha um papel essencial na ativação da imunidade adaptativa. É regulada a vários níveis, de modo a minimizar o grau de danos infligidos aos tecidos. As células apresentadoras de antigénio (CAA) constituem um desses níveis de regulação, uma vez que controlam a ativação das células T auxiliares (T_A), estabelecendo a ponte entre imunidade inata e adaptativa. A resposta gerada pelas células T_A é geralmente protetora. No entanto, em algumas circunstâncias, que não se compreendem ainda totalmente, pode tornar-se patológica, como acontece na esclerose múltipla (EM) e no seu modelo animal encefalomielite autoimune experimental (EAE). Desta forma, seria de esperar que mecanismos que limitem a ativação e/ou reativação de células T_A previnam a patogénese da neuroinflamação autoimune. Esta Tese descreve dois desses mecanismos: o primeiro é controlado pelo fator de transcrição, Fator 2 relacionado com o NF-E2 (Nrf2) e o segundo é controlado por um dos genes que aquele regula, o gene da enzima heme oxigenase-1 (*Hmox1*/HO-1). O Nrf2 regula a resposta celular ao stress oxidativo, enquanto a HO-1 é uma enzima que cataboliza grupos hémicos gerando monóxido de carbono (CO), ferro e biliverdina. Tanto o Nrf2 como a HO-1 foram previamente implicados na redução da resposta inflamatória, incluindo a que se encontra presente na neuroinflamação autoimune.

O objetivo desta Tese foi compreender o papel do Nrf2 e da HO-1 na regulação da neuroinflamação autoimune. Para isso, estudámos o papel da expressão do Nrf2 e da HO-1 na patogénese da EAE. Investigámos ainda, de um ponto de vista terapêutico, o impacto da indução farmacológica da expressão da HO-1 e da administração exógena de um dos seus produtos, o CO.

Ratinhos com disrupção funcional do gene que codifica o Nrf2 ($Nrf2^{-/-}$) desenvolvem uma forma de EAE mais severa do que ratinhos com o gene intacto ($Nrf2^{+/+}$). Este aumento de severidade é repetido quando se transferem leucócitos

encefalitogénicos adotivos de ratinhos *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{+/+}$ para ratinhos naïve. A expressão da interleucina (IL)-12 está aumentada em células dendríticas (CD) *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{+/+}$ no estado basal e após estimulação com LPS. Quando expostas a péptidos derivados da mielina, CD *Nrf2*$^{-/-}$ promovem a diferenciação de células T_A, reativas contra a mielina, em células T_A tipo 1 (T_A1), que produzem interferão (IFN)-γ. O efeito encefalitogénico dos leucócitos *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{+/+}$ é suprimido quando a atividade da IL-12/23p40 é neutralizada com um anticorpo monoclonal. O mesmo se verifica quando leucócitos encefalitogénicos adotivos de ratinhos *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{+/+}$ são transferidos para ratinhos naïve, com disrupção funcional do recetor do IFN-γ (*Ifngr1*$^{-/-}$). O Nrf2 inibe a produção de IL-12 em CD através de um mecanismo que envolve a expressão da HO-1. No entanto, ratinhos com disrupção do gene que codifica a HO-1, especificamente em CD desenvolvem uma forma de EAE semelhante à observada em ratinhos controlo. Em suma, a expressão do Nrf2 em CD contraria a patogénese da neuroinflamação autoimune através de um mecanismo que inibe a produção de IL-12 em CD, prevenindo assim a diferenciação de células T_A1. Este efeito protetor não parece ser mediado pela expressão da HO-1 em CD.

No entanto, ratinhos com disrupção funcional sistémica do gene da HO-1 (*Hmox1*$^{-/-}$) desenvolvem uma forma de EAE mais severa do que ratinhos com o gene intacto (*Hmox1*$^{+/+}$). A indução da HO-1 pela administração de protoporfirina IX de cobalto (CoPPIX), após o aparecimento dos sinais clínicos, resulta na melhoria da progressão da doença, e este efeito desaparece em ratinhos *Hmox1*$^{-/-}$, o que indica que o CoPPIX atua via HO-1 para suprimir a progressão da EAE. O efeito protetor da indução farmacológica da HO-1 está associado a uma inibição da expressão do complexo principal de histocompatibilidade classe II (CPHII) em células apresentadoras de antigénio, incluindo CD, macrófagos e microglia, e na consequente redução da acumulação, proliferação e função efetora das células T_A1 e T $CD8^+$ no sistema nervoso central. A administração exógena de CO simula este efeito, o que sugere que este gás contribui para a ação protetora da HO-1 em EAE. Em suma, o gene da HO-1 controla a severidade da EAE e a indução farmacológica da sua expressão regula negativamente a expressão do CPHII em

CAA, regulando assim a resposta T e a severidade da EAE, supostamente via produção de CO, podendo vir a ser usado para tratar a EM.

No seu conjunto, os resultados apresentados nesta Tese revelam que a expressão do Nrf2 e a indução farmacológica da expressão da HO-1 desempenham um papel na regulação de CAA, com impacto na resposta T que está na origem da neuroinflamação autoimune, previamente subestimado. Estes dados revelam a importância do Nrf2 e da HO-1 no controlo da neuroinflamação autoimune através da modulação de células da imunidade inata e consequentemente da imunidade adaptativa.

Abstract

Inflammation is a protective response generated by innate immune cells, upon infection and/or tissue injury, and aims at clearing the source of infection or noxious stimuli. It is required for activation of adaptive immunity. Inflammation has several layers of regulation in order to minimize the degree of tissue damage. Antigen-presenting cells (APC) constitute one layer of regulation as they initiate the activation of T helper (T_H) cells, bridging innate and adaptive immunity. The T_H cell response generated is usually protective, although in certain circumstances, that are not completely understood, can also become pathological, as occurs in multiple sclerosis (MS) and in its mouse model, experimental autoimmune encephalomyelitis (EAE). Thus, mechanisms restraining T_H cell activation and/or reactivation should prevent the pathogenesis of autoimmune neuroinflammation. In this Thesis we describe two of such mechanisms: one controlled by the transcription factor NF-E2-related factor 2 (Nrf2) and another controlled by one of its downstream genes *Heme oxygenase-1* (*Hmox1*), which encodes the enzyme HO-1. Nrf2 is a transcription factor that regulates cellular responses to oxidative stress, while HO-1 catabolizes heme into carbon monoxide (CO), iron, and biliverdin. Both, Nrf2 and HO-1 have been implicated in dampening inflammatory reactions, including autoimmune neuroinflammation.

The aim of this Thesis was to understand the role(s) of Nrf2, and its downstream gene *Hmox1*, in the regulation of autoimmune neuroinflammation. For this, we studied the role of *Nrf2* and *Hmox1* expression in the pathogenesis of EAE. Further, we investigated the impact of pharmacological induction of HO-1 expression and exogenous CO administration from a therapeutic point of view.

Nrf2-deficient ($Nrf2^{-/-}$) mice develop a more severe form of EAE, as compared to wild type ($Nrf2^{+/+}$) counterparts. This is recapitulated when encephalitogenic leukocytes from $Nrf2^{-/-}$ *vs.* $Nrf2^{+/+}$ mice are adoptively transferred into naïve recipients. Expression of interleukin (IL)-12 is increased in resting and LPS-activated $Nrf2^{-/-}$ *vs.* $Nrf2^{+/+}$ dendritic cells (DC). When "pulsed" with myelin-derived peptides, $Nrf2^{-/-}$ DC favor the differentiation of myelin-reactive T_H cells

towards a T_H1 phenotype, producing IFN-γ. The encephalitogenic effect of *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{+/+}$ leukocytes is suppressed when IL-12/23p40 activity is blocked using a monoclonal antibody. This is also the case when encephalitogenic leukocytes are adoptively transferred into naïve recipients lacking the IFN-γ-receptor. Nrf2 inhibits IL-12 production in DC via a mechanism that involves the expression of HO-1. Mice carrying a deletion of the *Hmox1* gene specifically in DC develop a similar form of EAE *vs.* wild type controls. Therefore, expression of Nrf2 in DC counters the pathogenesis of autoimmune neuroinflammation via a mechanism that inhibits IL-12 production in DC, preventing encephalitogenic T_H1 cell differentiation. This protective effect does not seem to be mediated by the expression of HO-1 in DC.

However, mice with systemic deletion of *Hmox1* (*Hmox1*$^{-/-}$) also develop a more severe form of EAE, as compared to control mice (*Hmox1*$^{+/+}$). Induction of HO-1, by cobalt protoporphyrin IX (CoPPIX) administration after EAE onset, reversed EAE progression and this effect was abrogated in *Hmox1*$^{-/-}$ mice, indicating that CoPPIX acts via HO-1 to suppress EAE progression. The protective effect of HO-1 induction was associated with inhibition of expression of MHC class II by DC, macrophages and microglia, and thus inhibition of accumulation, proliferation, and effector function of T_H and T_C cells within the CNS. Exogenous CO mimicked these effects, suggesting that CO contributes to the protective action of HO-1. Therefore, HO-1 controls the severity of EAE and its pharmacological induction negatively regulates the expression of MHC class II in APC and thus, the T cell response and severity of EAE. If, as we suggest, this effect is attributable to the production of CO, then treatment of MS might be possible through the therapeutic administration of CO.

In conclusion, the results presented in this Thesis reveal that Nrf2 expression, and pharmacological induction of HO-1, play a previously underappreciated role in the regulation of APC, with impact on the T_H cell response at the core of autoimmune neuroinflammation. These data reveals the importance of Nrf2 and HO-1 in controlling autoimmune neuroinflammation, via modulation of innate immune cells and consequently adaptive immunity.

Chapter 1

General Introduction

1. The immune response

Immunity comprises a set of mechanisms that provide host protection to infection, and it can be subdivided into innate and adaptive immunity[1].

1.1. Innate immunity and inflammation

Innate immunity is the first line of defense against infection and is characterized by the recognition of microorganisms, through germline encoded receptors, which detect evolutionarily conserved molecular patterns expressed by microbes. It is composed by a diverse set of cells and soluble mediators. Innate immune cells have hematopoietic origin, including dendritic cells (DC), macrophages (Mø), neutrophils, mast cells, basophils, eosinophils, NK cells and $\gamma\delta$ T cells[1]. Soluble mediators include collectins, ficolins and pentraxins[2].

Innate immune cells express multiple germline encoded receptors called pattern-recognition receptors (PRRs) with specificity to a large number of molecular structures shared among classes of pathogenic and commensal microorganisms, named pathogen-associated molecular patterns (PAMPs)[1,3]. Only a specific class of PAMPs, viability-associated PAMPs (VitaPAMPs), appears to induce immune responses[4]. PRRs can also recognize molecules released by injured or dying cells, termed damage-associated molecular patterns (DAMPs), such as adenosine triphosphate (ATP), potassium ions, uric acid, high-mobility group box 1 protein (HMGB1) and members of the S100 calcium-binding family of proteins[2]. However, it is not clear whether innate immune activation by DAMPs empower these cells with the ability to activate adaptive immunity (*see section 1.2*)[2].

PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG I-like receptors (RLRs)[3,5]. PRRs can be classified into secreted, transmembrane or cytosolic. Secreted PRRs include collectins, ficolins and pentraxins. These bind to microbial cell surfaces and activate the complement system[5], which consists of a series of circulating inactive proteins, many being proteases, that become themselves activated by proteolytic cleavage in sequential reactions, releasing effector peptides. The complement cascade can be initiated by three major pathways: Alternative, Classical and Lectin[6]. The Alternative

pathway is spontaneously and continuously activated and plays a crucial role in immune surveillance[7]. The Classical and Lectin pathways of complement activation are induced when antibodies bind to their corresponding antigen or when mannan-binding lectin binds mannose residues on the surface of microorganisms, respectively[6]. The process by which a microorganism is bound by a complement component facilitating its phagocytosis is called opsonization. Triggering of the complement cascade generates pro-inflammatory mediators, antimicrobial compounds, and anaphylactic peptides, and can lead to assembly of the multimeric protein membrane attack complex (MAC) that forms a cytolitic pore in the cell membrane[6].

Transmembrane PRRs comprise TLRs and CLRs located in cellular membranes and endosomes[5]. TLRs are the best characterized class of PRRs and are expressed in a cell-specific manner[8]. Cell-surface TLRs recognize PAMPs accessible on the cell surface, such as lipopolysaccharide (LPS) of Gram-negative bacteria (TLR4), while endosomal TLRs detect microbial nucleic acids such as double-stranded RNA (dsRNA; TLR3) or unmethylated CpG sequences common in bacterial and viral DNA (TLR9)[8]. CLRs localize in the outer membrane and detect carbohydrate components on fungal cell walls[9].

Cytosolic PRRs consist of NLRs and RLRs[5]. NLRs detect degradation products of peptidoglycans (a cell wall component of bacteria) and other microbial products, as well as several forms of stress and non-infectious crystal particles[10]. RLRs are expressed by virtually all cell types and detect viral nucleic acids[11].

Recognition of PAMPs or DAMPs by PRRs triggers the activation of specific signal transduction pathways and ultimately leads to the expression of effector molecules[12,13] (*Fig.1*). These comprise cytokines, chemokines, vasoactive amines, lipid mediators and products of several proteolytic cascades, such as the complement system[2].

Tissue-resident innate immune cells play a crucial role in detecting the presence of pathogens as well as tissue injury[1]. Engagement of PRRs in tissue resident Mø and mast cells leads to their activation and initiation of inflammation, a response aimed at clearing pathogens and restoring tissue homeostasis[2]. In

addition, DC activation by PRR is crucial for the generation of adaptive immunity, as discussed in detail below (*see section 1.1.1 and 1.2.1.2*).

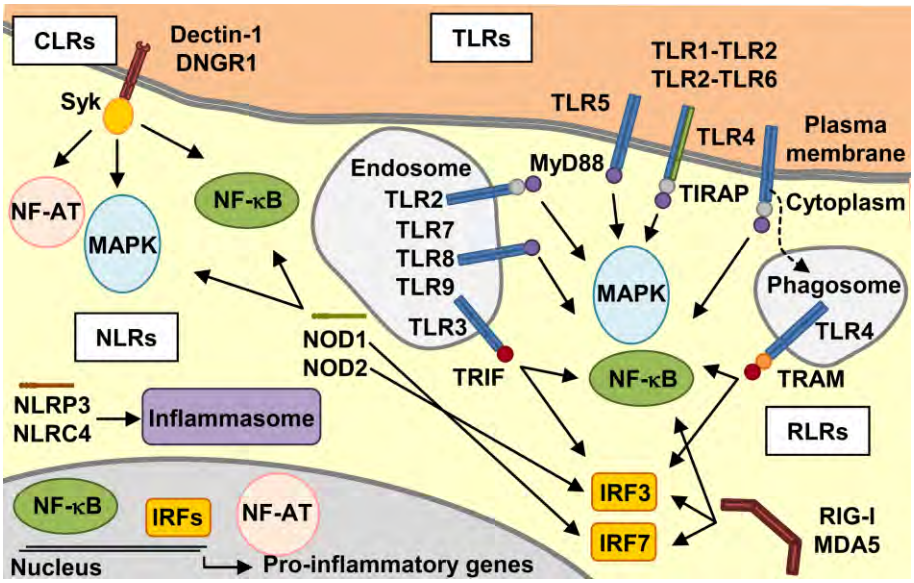

Figure 1. Signal transduction pathways triggered by PRR engagement. TLR engagement leads to homo or heterophylic interactions and to the TLR-specific recruitment of the intracellular signaling adaptor molecules: myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and TIR-associated protein (TIRAP)[8]. TIRAP and TRAM are adaptor molecules that allow MyD88 and TRIF recruitment, respectively. The adaptors recruited are specific for the TLR triggered, i.e. TLR4 can recruit the four adaptors, while TLR3 only TRIF and TLR9 only MyD88[12]. TLR signaling via MyD88 culminates in the activation of MAPK and NF-κB and via TRIF in the activation of NF-κB and IRF-3[12,13]. CLRs (i.e. Dectin-1, DNGR1, Dectin-2) signal, either directly or by recruiting adaptor proteins, via spleen tyrosine kinase (Syk) leading to the activation of MAPK, NF-κB and NF-AT[12,13]. NLRs activate MAPK and NF-κB as well as IRF-3 (NOD2) and IRF-7 (NOD1). NLR family members such as NLRP3 and NLRC4 form multiprotein complexes, the inflammasomes, leading to the activation of pro-inflammatory caspases, generating active cytokines from inactive precursors[10]. RLRs (e.g. RIG-I and MDA5) lead to the activation of NF-κB, IRF-3 and IRF-7[12,13].

The inflammatory response is revealed clinically by four classical signs described by Celsus in AD40 as "*calor, rubor, tumor* and *dolor*". However, it is currently believed that asymptomatic inflammatory responses of lower magnitude occur more frequently than appreciated and play a central role in maintaining tissue homeostasis[2]. Presumably, inflammation, or a related process termed para-inflammation, sharing many effector molecules, plays a critical physiological role in restoring homeostasis after mild cellular stress and tissue dysfunction, occurring in many instances irrespectively of infection[2].

The kinetics of the inflammatory response comprises three distinct phases: *initiation*, *amplification* and *resolution*, each characterized by the expression of different mediators. PRR engagement triggers the *initiation* phase of the inflammatory response resulting in the coordinated delivery of circulating leukocytes, mainly neutrophils, and plasma proteins at a site of microbial infection or injury, while providing a physical barrier that prevents spreading of the infectious or noxious agent. Neutrophils gain access to the tissue through the endothelium and are activated via PRRs[14]. This leads to the release of the toxic contents of their granules such as reactive oxygen and nitrogen species (ROS and RNS, respectively), proteinase 3, cathepsin G and elastase[14]. If these toxic molecules are not sufficient to eliminate the infection, an additional layer of control is provided via activation of adaptive immunity (*see section 1.2*). This is called the *amplification* phase.

While inflammatory mediators can eliminate the infection, they also cause tissue damage to the infected host. Once the infectious agent has been cleared, or the cause of tissue injury eliminated, a phase of repair and healing ensues, and this is called *resolution*. It avoids the development of pathological states characterized by chronic inflammation and activation of adaptive immunity, avoiding irreversible tissue damage. Failure in resolution of inflammation leads to the development of immune-mediated inflammatory diseases, acting as major causes of mortality and morbidity worldwide[15]. These include autoimmune diseases, such as multiple sclerosis (MS) in which tissue damage occurs within the central nervous system (CNS) (*see section 2*).

During the *resolution* phase of an inflammatory response, apoptosis of neutrophils is triggered, while that of neighboring parenchyma cells is avoided. Recognition of apoptotic cells switches the cytokine and lipid mediators produced by resident Mø from a pro- to anti-inflammatory profile[16,17]. Examples include the anti-inflammatory cytokines interleukin (IL)-10[16] and transforming growth factor (TGF)-β[17] as well as lipid mediators, such as lipoxins, resolvins and protectins[18]. Numerous other mechanisms contribute to the dampening of inflammation including but not limited to: i) inhibition of the major signal transduction pathways triggered by

PRRs, ii) suppression of innate immune responses by adaptive immune cells, iii) suppression by regulatory T cells (T_{REG}), iv) control of lymphocyte expansion and promotion of cell death, and v) expression of anti-inflammatory stress-responsive genes (*see section 3.3 and 3.3.1*).

1.1.1. Dendritic cells (DC)

The main function of DC is the recognition of pathogens and the subsequent priming of adaptive immune responses, against specific antigens expressed by those pathogens[19]. Nonetheless, DC are also critical in suppressing immune responses, maintaining peripheral tolerance and tailoring T cell responses, according to the pathogen encountered[20,21].

DC are the most efficient antigen-presenting cells (APC) in the immune system[22], capturing, processing and presenting antigens in the context of major histocompatibility complex (MHC) to T cells[19]. DC have the unique ability among all other APC of activating naïve T cells, bridging innate and adaptive immune systems, and are therefore called professional APC[23]. Other cells, such as Mø and B cells, can act as APC but mainly to reactivate primed T cells and thus only amplify the response[23]. Antigen presentation by DC can either be immunogenic or tolerogenic[20,21]. When immunogenic, DC trigger T cell proliferation and differentiation into distinct effector subsets (*see section 1.2.1.2; Fig.2b*), while tolerogenic DC promote T cell deletion, anergy or regulatory function[20] (*Fig.2a*). The DC maturation status, DC subset, environment and the balance between pro- and anti-inflammatory gene expression determines whether a DC-T cell interaction will be immunogenic or tolerogenic[21].

DC are defined phenotypically by their specific morphology, characterized by the presence of dendrites, as well as by the surface expression of MHC class II and of the integrin cluster of differentiation (CD)11c[24]. Nevertheless, DC constitute an heterogeneous population composed of many functional subsets based on their location, surface marker expression and whether or not they are present during homeostasis[24,25]. All DC subsets are thought to have a common hematopoietic origin and to reside in tissues, such as the skin and mucosal surfaces, where they

sample the environment and capture antigen, and in secondary lymphoid organs, such as the spleen and lymph nodes (LN)[26]. Splenic DC can be subdivided into plasmacytoid or conventional DC[24]. Plasmacytoid DC possess the unique feature of quickly secreting large amounts of type I interferons in response to PRR engagement[27]. Conventional DC can be further subdivided based on the surface expression of CD8α into CD8α^+ and CD8α^- cells[24]. These latter can be sub-classified based on CD4 expression, but the physiologic relevance of this classification is still not clear[24].

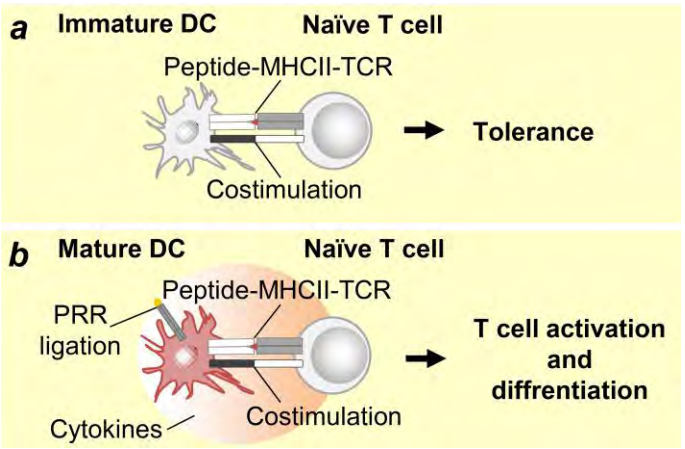

Figure 2. DC maturation status dictates the result of the DC-T cell interaction. (***a***) Under homeostatic conditions, DC display an immature phenotype, expressing low levels of MHC class II and costimulatory molecules (e.g. CD40, CD80 and CD86)[28]. They are characterized by a high endocytic and phagocytic capacity[28], sampling antigens and migrating to lymph nodes or the spleen thereafter where they present antigens to T cells[19,21]. Here, immature DC-T cell interactions result in suboptimal priming, favoring T cell tolerance[21]. (***b***) Signals such as PAMPs, DAMPs or pro-inflammatory cytokines can trigger DC maturation[21]. These signals cause a transient increase in antigen uptake and processing by DC, that subsequently decreases as DC mature, and is accompanied by the up-regulation of MHC class II and costimulatory molecules[28]. Mature DC present antigens via MHC class II for T cell receptor (TCR) recognition, providing costimulation, which will trigger T cell activation and proliferation[19,28]. Costimulation and secretion of a variety of cytokines will influence T cell differentiation into distinct effector T_H cell subsets (*see section 1.2.3*)[19,34].

In vivo, DC can exist in two states, immature and mature and only mature DC are able to prime immune responses[28] (*Fig.2b*). A mature DC is defined by its ability to act in a immunogenic manner, i.e. to elicit an adaptive immune response,

upon presentation of antigen in the context of MHC molecules to naïve T cells[28]. Importantly, PAMPs, but not DAMPs can generate immunogenic DC.

1.2. Adaptive immunity

Adaptive immunity provides a highly specific response against infectious pathogens, while generating immunological memory, which refers to a quantitatively and qualitatively improved immune response upon subsequent exposure to the same antigen[13]. Contrary to innate immunity, the adaptive immune system relies on clonally expressed and highly specific antigen receptors, capable of recognizing virtually any antigen[13]. The high specificity and affinity of antigen receptors is due to somatic recombination and hypermutation of the genes encoding these receptors. These are composed of several segments that can be assembled in different combinations in a process called somatic recombination. In addition, a controlled program of creating point mutations in these genes generates high-affinity antigen receptor variants in a process called somatic hypermutation.

Antigen receptors are called B cell- and T cell-receptors (BCR and TCR), when expressed on B and T cells, respectively, the two cell types that compose the cellular arm of the adaptive immune system[1]. B and T cell precursors originate in the bone marrow, with B cell precursors maturing in the bone marrow while T cell progenitors migrate to the thymus where they give rise to mature T cells. The adaptive immune system is also composed by a humoral arm, provided by soluble BCR, i.e. antibodies produced by activated B cells that differentiate into antibody-producing plasma cells. Antibodies belong to different immunoglobulin (Ig) classes/isotypes that have different effector functions.

B cells can be activated in a T cell-dependent or independent manner, resulting in differentiation into plasma cells that mediate the responses through antibody production. On the other hand, T cells mediate their function through the production of pro-inflammatory cytokines or cytotoxic activity.

1.2.1. T cells

T cells can be subdivided into T cytotoxic (T_C), helper (T_H) and T_{REG} cells[1]. T_C are defined by the expression of the TCR co-receptor CD8 whereas T_H and T_{REG} by the expression of CD4. T_C cells recognize antigens presented in the context of MHC class I while T_H and T_{REG} cells do so in the context of MHC class II molecules[29]. T_C cells mediate defense, mainly against viral infections and tumors, by virtue of their direct cytotoxic effect exerted on target cells in an antigen-specific manner[30]. T_H cells orchestrate immune responses against a wide range of pathogens, help B cells produce antibodies, enhance and maintain T_C cell responses, regulate Mø function[31]. T_{REG} suppress immune responses to control autoimmunity and adjust the magnitude and persistence of the response[31].

1.2.1.1. T_H and T_{REG} cell lineages

There are at least four $CD4^+$ T cell lineages: T_H1, T_H2, T_H17, and T_{REG} (*Fig.3*). T_H1, T_H2 and T_H17 are important for eradicating intracellular pathogens, helminths and extracellular bacteria/fungi, respectively[31]. In addition, T_H2 cells contribute to allergic responses, whilst T_H1 and T_H17 play an important role in the pathogenesis of autoimmune diseases, such as MS (*see section 2*)[31]. T_{REG} cells suppress immune responses and maintain peripheral tolerance (*see section 1.3.2*)[32]. There are several described populations of regulatory T cells, such as Tr1, Th3, but the best characterized are T_{REG}, defined by the surface expression of IL-2 receptor α chain (CD25) and the transcription factor forkhead box P3 (Foxp3)[31,33]. T_{REG} can be subdivided in two subsets based on how they are generated. Natural T_{REG} (nT_{REG}) are generated in the thymus, expressing Foxp3 constitutively, while inducible T_{REG} (iT_{REG}) are generated in the periphery by the induction of Foxp3 expression upon antigen encounter[32] (*see section 1.2.1.2*).

Two other T_H cell phenotypes may be considered as lineages, i.e. T_H9 and follicular helper T (T_{FH}) cells. T_H9 cells are derived from T_H2 cells in the presence of TGF-β and it is unclear whether they constitute a new lineage or are the result of adaptation of T_H2 cells to a change in the microenvironment during an ongoing immune response[31,34]. T_{FH} cells reside in B cell follicles where they provide help to

B cells. T_{FH} can secrete T_H1, T_H2 and T_H17 signature cytokines, which raises questions as to whether this is a *bona fide* T cell lineage or an adaptation of these subsets to the environment in B cell follicles[31,34].

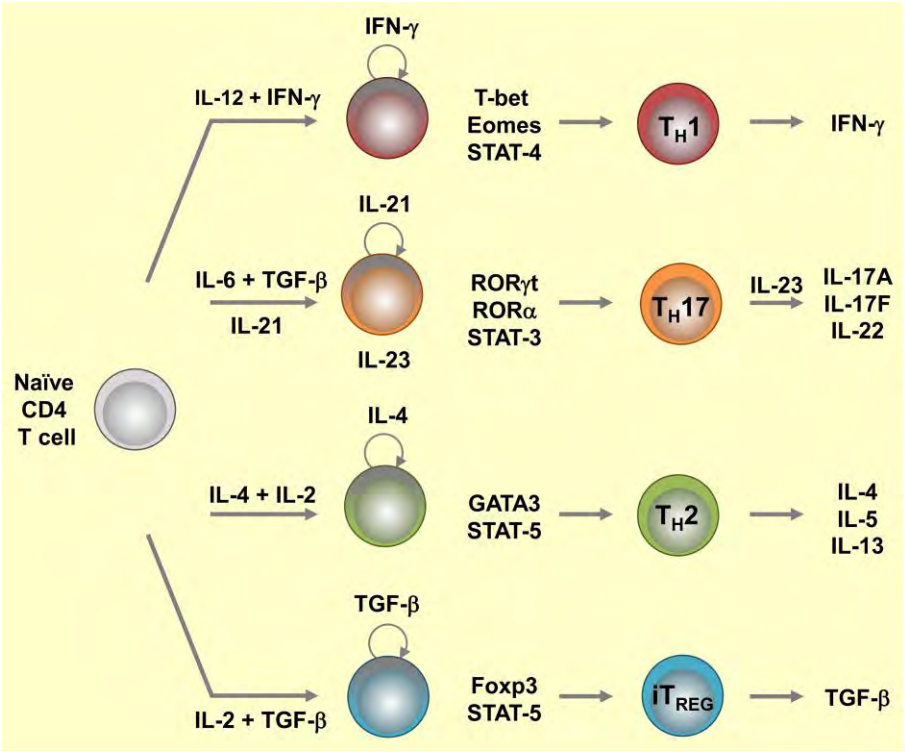

Figure 3. Generation of effector T_H and iT_{REG} cell subsets. Cytokines play critical roles in the differentiation and effector functions of T_H1, T_H17, T_H2 and iT_{REG} cells. Upon TCR activation triggered by DC, naïve $CD4^+$ T cells differentiate into distinct lineages in the context of different combinations of cytokines. The differentiation process involves the activation of specific STAT proteins and up-regulation of lineage-specific transcription factors. Each lineage then secretes a specific pattern of cytokines.

Effector T_H and iT_{REG} cell lineages produce specific patterns of cytokines that relate to the particular function of that lineage (*Fig.3*). For T_H1 cells, interferon (IFN)-γ is the signature cytokine. T_H1 cells also produce lymphotoxin[31,35] as well as high levels of IL-2 and tumor necrosis factor (TNF)[31,35]. T_H1 cytokines are potent activators of Mø, which deal with intracellular pathogens[36]. T_H2 cells secrete IL-4, IL-5 and IL-13 as signature cytokines[31,35]. They also produce IL-10, TNF, modest amounts of IL-2 and some can produce IL-9[31]. T_H2 cytokines are critical for IgE

production, eosinophil recruitment and clearance of extracellular parasites[30,35,37]. T_H17 cells are characterized by the production of IL-17A, IL-17F and IL-22 as well as high levels of IL-21[31,38-40]. These induce the recruitment of neutrophils and clear extracellular bacterial and fungal infections[41]. iT_{REG} produce TGF-β as signature cytokine and limit the magnitude of immune responses[31,42].

1.2.1.2. Effector T_H and iT_{REG} cell generation

Upon TCR engagement, a combination of cytokines is required for the differentiation of each T_H cell subset and iT_{REG} and generation of effector function: IL-12 and IFN-γ for T_H1, IL-4 and IL-2 for T_H2, TGF-β and IL-6/IL-21/IL-23 for T_H17 and TGF-β and IL-2 for iT_{REG}[31] (*Fig.3*). In all cases, the effector cytokines produced by a given lineage promote further differentiation into that subset, via a positive feedback loop that stabilizes lineage commitment. This appears to be the case for T_H1 via the production of IFN-γ, for T_H2 via the production of IL-4, for T_H17 via the production of IL-21, and for iT_{REG} via the production of TGF-β[31]. At later stages of T cell differentiation, TCR-independent cytokine production can also be induced by IL-18 for T_H1, IL-7/ thymic stromal lymphopoietin (TSLP)/IL-33 for T_H2 and IL-1 for T_H17[31].

Cytokines and costimulatory molecules produced by innate immune cells, and in particular by DC, play a critical role in determining $CD4^+$ T cell differentiation towards T_H1, T_H2, T_H17 or iT_{REG}. IL-12 produced by DC is necessary and sufficient for T_H1 differentiation both, *in vitro*[43] and *in vivo*[44], as demonstrated in the context of *Toxoplasma gondii* infection. Expression of the costimulatory molecule CD70 by DC also induces T_H1 differentiation[45,46]. In addition, different subsets of DC can induce specific T_H cell lineages. Splenic $CD8\alpha^+$ DC that localize preferentially in the T cell rich areas of the spleen secret high levels of IL-12, driving T_H cell activation towards a T_H1 phenotype[24,47,48]. In contrast, $CD8\alpha^-$ DC are localized in the marginal zone of the spleen and do not generally secrete much IL-12, secreting preferentially IL-10, and as such, inducing T_H2 responses[24,47,48]. The contribution of DC-derived cytokines for the differentiation of other T_H cell lineages is less clear. Whether

cytokines necessary for T_H2, T_H17 and iT_{REG} differentiation are DC-derived is still poorly defined[24]. In addition, other innate immune cells can influence T_H cell differentiation by producing cytokines that favor commitment towards specific lineages. γδ T cells promote T_H17 responses via the production of IL-21, basophils T_H2 via the production of IL-4 and NK cells T_H1 via the production of IFN-γ[34].

1.2.1.3. Molecular mechanisms of effector T_H and iT_{REG} cell generation

Signaling by several cytokines, including IL-12, IFN-γ, IL-4, IL-6, IL-21, IL-23 and IL-2 trigger the activation of signal transducer and activator of transcription (STAT) proteins and the expression of transcription factors essential for T_H and iT_{REG} cell lineage commitment[31]. In addition, these cytokines are also involved in active suppression of alternative lineage fates. This occurs via the regulation of genes promoting lineage commitment, while repressing alternative lineage genes, therefore rendering subsets mutually exclusive. STAT-4, T-box transcription factor (T-bet) and Eomesodermin are important for T_H1 differentiation, STAT-5 and trans-acting T cell-specific transcription factor (GATA) 3 for T_H2, STAT-3, retinoic acid receptor-related orphan receptor (ROR) γt and α for T_H17, and STAT-5 and Foxp3 for iT_{REG}[31] (*Fig.3*). These transcription factors bind directly to regulatory DNA regions, such as promoters, enhancers, insulators and locus control regions of the effector cytokine genes, i.e. T-bet for *Ifng*, GATA3 for *Il4* and *Il13* and RORγt for *Il17a* and *Il17f* [31], inducing gene activation, repression or epigenetic modifications, thus regulating gene expression[31]. This phenomenon is well characterized for T_H1 lineage commitment, where the transcription factor T-bet promotes IFN-γ transcription[49] by chromatin remodeling at the *Ifng* gene promoter locus[50].

There is a great degree of plasticity between $CD4^+$ T cell lineages. Several examples have been described, for example for iT_{REG} and T_H17 differentiation plasticity[51]. Briefly, TGF-β induces both Foxp3 and RORγt in naïve $CD4^+$ T cells, but Foxp3 is dominant and antagonizes RORγt function unless IL-6 is present[52]. Thus, an inflammatory environment alters the balance between iTreg and T_H17 differentiation towards the T_H17 lineage. T_H17 differentiation *in vitro* also shows a

STAT4- and T-bet-dependent plasticity towards T_H1 and *in vivo* during chronic inflammation T_H17 cells convert to IFN-γ producers[34,53-55]. T_H2 cells can produce both IL-4 and IFN-γ upon a viral infection *in vivo*[56] and IL-10 can be induced in T_H1 cells during infection, generating cells that produce both IL-10 and IFN-γ[57,58]. These observations suggest that $CD4^+$ T cell lineage commitment *in vivo* is probably not as rigid as it appears from *in vitro* studies. There is flexibility in the secretion of cytokines among T_H and iT_{REG} lineages, according to the trigger and phase of the immune response, conferring adaptability to this response. However, the plasticity appears not to be random but to follow well-defined rules.

1.3. Immune tolerance

The immune system maintains a delicate balance between responding to non-self, pathogen-derived, antigens, while acquiring and maintaining a state of tolerance to the body's self-antigens, commensal microorganisms and foreign innocuous antigens[59]. The disturbance of this balance can lead either to pathologic tolerance in detriment of immunity, resulting in chronic infections and tumors, or a pathologic failure of tolerogenic mechanisms resulting in autoimmune diseases, such as MS[59] (*see section* 2). Immune tolerance is maintained by central and peripheral mechanisms. Central tolerance operates in the thymus and in the bone marrow to eliminate self-reactive immature T and B cells, respectively[59], while peripheral tolerance operates once mature T and B cells emigrate from the thymus or bone marrow, and controls auto-reactive T and B cells in the periphery[60].

1.3.1. Central T cell tolerance in the thymus

During T cell development in the thymus, thymocytes are selected by thymic APC presenting self-antigens representing the diversity of peripheral tissues[61]. Positive selection is the process by which thymocytes that are able to establish intermediate affinity interactions with a peptide-MHC complex receive survival signals, while those with no or very low affinity undergo apoptosis[61,62]. Negative selection consists in the elimination by apoptosis of thymocytes with high affinity for self-antigens[61].

This process aims at purging the mature T cell repertoire from self-reactive T cells with a high affinity towards self-antigens, as to prevent the development of autoimmune diseases, such as MS (*see section 2*).

However, central tolerance mechanisms are not 100% effective and as a consequence, self-reactive T cells escape and are found in the peripheral T cell repertoire of healthy individuals[61,62]. Peripheral tolerance mechanisms are thus essential to control the activation of these auto-reactive T cells and prevent T cell driven autoimmunity, such as occurs in MS (*see section 2*).

1.3.2. Peripheral T cell tolerance

Peripheral tolerance mechanisms comprise inactivation of auto-reactive T cells by deletion, induction of anergy or suppression by T_{REG} cells[21,60]. iT_{REG} cells play a pivotal role in the maintenance of peripheral tolerance[63], via a mechanism involving the production of IL-10 and TGF-β. The suppressive activity of nT_{REG} is thought to rely also on cell/cell-contact and inhibitory surface molecules targeting DC and T cell activation[64]. Several mechanisms have been suggested and can be grouped into: 1) suppression by cytolysis, acting via granzyme secretion or the TNF-related apoptosis inducing ligand death receptor 5 (TRAIL-DR5) pathway; 2) metabolic disruption, acting via IL-2-deprivation-mediated apoptosis or through the release of adenosine nucleosides and 3) modulation of DC maturation or function, acting via the costimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4)[64]. Whether these mechanisms are redundant, or not, remains to be established. What is clear from the autoimmune phenotype associated with Foxp3 inactivation, in both mice and humans, is that T_{REG} cells are essential in maintaining immune homeostasis preventing autoimmune diseases, such as MS[33].

2. Multiple sclerosis (MS)

MS is the most prevalent chronic autoimmune inflammatory disease targeting the CNS[65-67]. It is the leading cause of neurological disability in young adults, affecting about one in 1000 individuals in Western countries and more than one million

people worldwide. Notably, it is two to three times more prevalent in women than in men[67].

It is a heterogeneous disease in terms of clinical symptoms, as well as progression (*Fig.4a*). Symptoms include defects in sensation and in the motor, autonomic, visual and cognitive systems[67,68]. In the early stages, around 85-90% of the individuals have a relapsing-remitting disease course (RR-MS), with slow-progressing disability, characterized by recurrent unpredictable episodes of neurological deficits followed by periods of remission where the symptoms disappear partially or completely[67,68] (*Fig.4a*). However, with time, about 50% of the individuals with RR-MS develop a progressive form of disease with cumulative and incapacitating disability, called secondary progressive multiple sclerosis (SP-MS)[67,68] (*Fig.4a*). 10-15% of the MS patients present at diagnosis a progressively worsening form of disease, called primary progressive (PP-MS)[67,68] (*Fig.4a*).

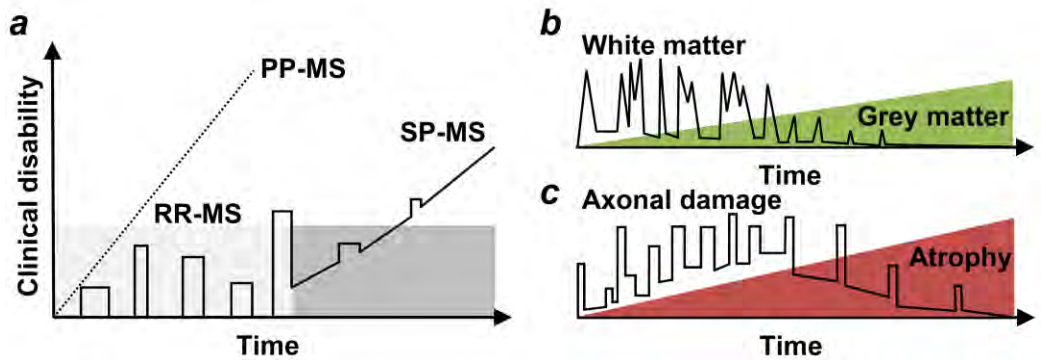

Figure 4. Progression of clinical disability in MS. (***a***) In PP-MS clinical disability increases cumulatively from the onset of MS. In patients with RR-MS, after onset of disease, sporadic clinical relapses are followed by periods of remission, until disease progression turns SP-MS where clinical disability increases cumulatively with no recovery. (***b***) In RR-MS acute white matter demyelination predominates at initial stages of the disease and declines with time, while grey matter demyelination is present from the beginning but increases in SP-MS.(***c***) In RR-MS acute axonal damage prevails in the first phase of the disease and is partially reversible. Brain atrophy starts from the onset of the clinical symptoms but increases in later stages of MS. *Adapted from Siffrin et al. (2010) Trends Neurosc.*

2.1. Pathology

MS pathology is clinically and histologically heterogeneous[66]. It has become increasingly recognized that MS may not refer to a single disease, but rather to a composite of several pathological processes manifested in different clinical

phenotypes that encompass RR-MS and SP-MS[67] (*Fig.4a*). MS is characterized by lesions in the white matter of the CNS composed of myelinated axons, as visualized by magnetic resonance imaging. These lesions are usually characterized by perivascular lymphocyte, Mø and antibody-producing plasma cell infiltration. They present signs of demyelination, oligodendrocyte injury and death, axonal damage and loss, as well as an increase in microglia and astrocytes, i.e. glial scar[69]. Antibody deposition and complement activation have also been reported[69]. It is widely accepted that T cells are at the core of the pathogenesis of MS[65,69] (*see section 2.4.3.2*). Immunosuppression studies support this notion, as neurological deficits associated with MS improve with treatment[70] and in particular with monoclonal antibodies targeting T cells[71].

The classical view of MS pathology emphasized the importance of CNS lesions in the white matter, leading to demyelination and oligodendrocyte loss, but recent evidence from histology and advanced MRI techniques revealed that grey matter (part of the CNS consisting of neuronal cell bodies and dendrites, glial cells and vasculature) pathology, causing neuronal death, is present from the earliest stages of MS (*Fig.4b*). In fact, cortical lesions tend to have fewer infiltrating leukocytes and more prominent neuronal degeneration than white matter lesions. Another feature of grey matter pathology is atrophy, which worsens more rapidly than white matter atrophy and correlates with disability[66] (*Fig.4c*). The current view is that MS pathology is complex, involving both immune and degenerative mechanisms[66,67]. Immune mechanisms target both white and grey matter and neurodegenerative processes include loss of axons in lesions, diffuse damage to white matter distant from areas of visible active lesions, and involvement of both deep and cortical grey matter[67,72].

The mechanisms underlying the heterogeneity of MS manifestations are still not clear, but the different localization and types of lesions among individuals with MS, as well as the different contributions of the immune and degenerative processes controlling MS pathogenesis could be part of the explanation.

2.2 Ethiology

The exact cause of MS is unknown, but epidemiological studies indicate that genetic and environmental factors, and their interaction, play a determinant role in disease predisposition[66,73,74].

2.2.1. Genetic factors

The case for genetic factors is strongly suggested by the observation of disease clustering in families and concordance rate studies in twins. Mono and dizygotic twin studies show a 5% concordance rate for the first and 25-40% for the latter, in both cases superior to the 0,1% prevalence of MS observed in Western populations[74,75]. In addition, genome-wide linkage analysis, candidate gene and genome-wide association studies revealed several genes that confer susceptibility to MS. The human leukocyte antigen (HLA) locus confers a dominant risk of developing MS, in particular the HLA-DRB1 gene[74]. HLA-DRB1*1501 is the main susceptibility allele, and risk conferred by this allele can still be increased or reduced depending on whether individuals carry in *trans* (in another chromosome) the alleles HLA-DRB1*0801 or HLA-DRB1*1401, respectively. In the HLA class I region, alleles that confer moderate to high risk (HLA-A*0301) or protection (HLA-A*0201 and HLA-C*05) have also been reported[74]. In addition, 49 other genes have alleles that confer a small risk to MS. These include, but are not limited to, immunological genes important in T cell responses that encode: cell adhesion molecules, costimulatory molecules, cytokines, cytokine receptors and signal transducers of these cytokine receptors[76]. Among these are polymorphisms in the human *IL12A* (*IL12p35*), *IL12B* (*IL12/23p40*), *IL12RB1*[76] and *IFNG*[77] genes, suggesting a pathogenic role for T_H1 cells in autoimmune neuroinflammation (*see section 2.4.3.2*).

Given the above, MS is considered a polygenic disease where many genes have small contributions to disease predisposition, and the pathologic outcome will depend on the genetic background of the individual and its interaction with post-genomic regulatory events, such as gene rearrangement, epigenetic modifications, RNA editing and splicing, and with environmental factors[73,74] (*Fig.5*).

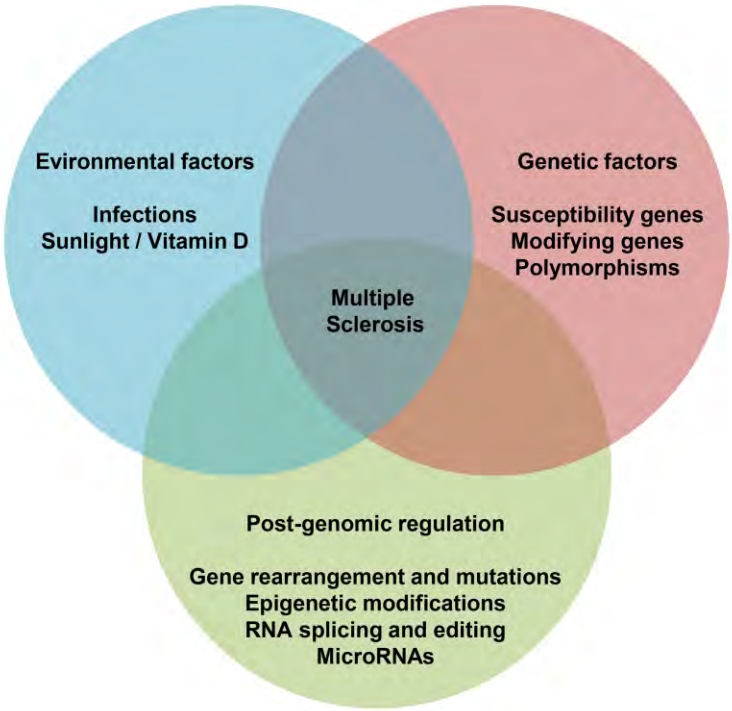

Figure 5. Factors that confer susceptibility to MS. The onset of MS results from genetic factors, responsible for a modest disease risk, and their interaction with post-genomic regulatory and environmental factors. For a matter of simplification only some of the factors in each category are mentioned. *Adapted from Oksenberg et al. (2010) Nat. Rev. Neurol.*

2.2.2. Environmental factors

The contribution of environmental factors to the pathogenesis of MS is revealed by the observation that monozygotic twins have only 25-40% instead of 100% concordance in MS development, with concordance rates differing by latitude. Migration, latitude, and month of birth all influence disease prevalence, further supporting a role for the environment in MS risk[73]. Reduced sunlight exposure is a candidate risk factor for the increase in MS prevalence with latitude. Sunlight is required for Vitamin D synthesis and low levels Vitamin D are common in individuals with MS, correlating negatively with relapse rate and disability. Preliminary studies also suggest high-dose Vitamin D supplementation may reduce MS relapses[66,73]. However, sunlight and Vitamin D seem to have independent effects on MS susceptibility[66,73] (*Fig.5*).

Several viral and bacterial infections have also been suggested to act as risk factors in MS, being the most compelling evidence provided for Epstein Bar virus (EBV) infection[66]. However, as for other infectious triggers a causal relationship is still missing[66]. Infection can trigger autoimmunity by several processes such as: i) molecular mimicry, ii) bystander activation or iii) epitope spreading. Molecular mimicry is the process by which autoreactive T cells cross-react with a microbial epitope sharing sequence or structural homology with a self-antigen[78]. Bystander activation refers to the non-antigen specific activation of autoreactive T cells by the inflammatory environment triggered, for instance, by an infection[78]. Epitope spreading refers to the development of reactivity to epitopes distinct from, and non-cross-reactive with, the disease-inducing epitope that is promoted during chronic inflammation[78] (*see section 2.4.1*; *Fig.5*).

2.3. Experimental models of MS

Much of the current understanding of the pathogenesis of MS is driven from its animal models. As it is still not clear whether MS is the result of an autoimmune response, a response to infection, or a combination of the two, the animal models available reflect this, with two types available: autoimmune and viral[79,80]. We will only discuss the autoimmune model, experimental autoimmune encephalomyelitis (EAE), as viral models fall out of the scope of this Thesis.

2.3.1 Experimental autoimmune encephalomyelitis (EAE)

The gold standard to study the autoimmune ethiology of MS is EAE[81,82]. The pathological mechanisms involved in producing the acute lesion of EAE share similarities with those of MS, making it a suitable model to understand the immunological basis of MS[79]. EAE can be actively induced in susceptible inbred mouse strains by immunization with spinal cord homogenate, myelin basic protein (MBP), proteolipid protein (PLP) or peptides from encephalitogenic regions of MPB, PLP or myelin oligodendrocyte glycoprotein (MOG)[83] in complete Freund's adjuvant (CFA)[79,80]. Depending on the mouse genetic strain and encephalitogen used, the profile of disease can vary from acute monophasic to chronic progressive or

relapsing-remitting[79]. MOG is located at the surface of the myelin sheath and is a minor myelin component[68,80]. When immunized with the MOG_{35-55} peptide in conjunction with *Pertussis* toxin (PTx) administration, C57BL/6 mice develop a chronic progressive EAE after an asymptomatic period of 10 to 20 days[80,83]. PTx facilitates T cell access to the CNS, presumably via TLR4-mediated induction of adhesion molecules in the endothelium of the blood-brain barrier[84]. In addition, PTx contributes to the break of T cell tolerance, promoting clonal expansion and cytokine production by T cells[79,80]. This immunization model promotes the activation of T_H cells, specifically of the T_H1 and T_H17 lineages[79,80]. CNS lesions are located predominantly in the spinal cord, leading to a classical EAE manifestation, that is a progressive ascending paralysis starting from the tail and reaching the forelimbs in the more severe stages[79].

Alternatively, EAE can be induced by adoptive transfer of T_H cells from immunized donors to naïve mice[85]. Adoptive transfer of different T_H cell lineages can cause autoimmune neuroinflammation[65,86] but of major importance are T_H1 and T_H17 lineages[87,88]. T_H1- and T_H17-driven EAE have functional and pathogenic differences[89,90]. T_H1-induced EAE causes classical EAE, in which infiltrating cells are mostly of monocytic origin and lesions are restricted to the spinal cord[89]. Instead, T_H17 cells cause atypical EAE that manifest as an axial rotatory movement that starts as a subtle head tilt and in more severe states leads to rotation over the longitudinal axis. The infiltrate is granulocytic and the lesions locate predominantly to the brain, brainstem and cerebellum[89]. In addition, the ratio of T_H1 *vs.* T_H17 cells determines the localization of the lesions[91]. Only when IL-17-secreting T_H cells are in excess over IFN-γ-producing cells, do EAE lesions localize to the brain, suggesting that IFN-γ signaling has a protective role in the brain, while pathogenic in the spinal cord[91,92]. Functional differences between T_H1 *vs.* T_H17 EAE are also illustrated by the fact that treatment with IFN-β improves T_H1 while worsening T_H17-induced pathology[91].

Several $CD4^+$ TCR transgenic mouse models recognizing specific myelin-derived peptides (MBP_{Ac1-9}, $PLP_{139-151}$, MOG_{35-55}) including a “humanized mouse”

model expressing a TCR specific for human MBP in the context of HLA-DR2 have been generated[79,80]. Importantly, in these models, development of spontaneous EAE occurs with variable incidence[93-96]. MBP_{Ac1-9} $CD4^+$ TCR transgenic develop spontaneous EAE with a frequency of 14%[93]. EAE incidence increases to 100% when MBP_{Ac1-9} $CD4^+$ TCR transgenic are crossed with recombination activating gene (*Rag1*)-deficient mice[93], in which endogenous TCR recombination is suppressed. This reveals that the remaining polyclonal T cell repertoire of *Rag1*-competent MBP_{Ac1-9} $CD4^+$ TCR transgenic mice exerts a potent regulatory effect over pathogenic anti-MBP_{Ac1-9} TCR transgenic T_H cells. This regulatory effect was later found to be mediated by nT_{REG} cells[97,98]. In a similar manner, MOG_{35-55} TCR transgenic mice (2D2) develop EAE at a frequency of 4%, but have a spontaneous incidence of optic neuritis of 30%[96]. Whether spontaneous incidence of EAE or higher incidence of optic neuritis increases when T_{REG} are deleted in 2D2 mice is likely to be the case, but this remains to be tested. Interestingly, for a significant proportion of individuals with MS, optic neuritis is the first clinical manifestation of the disease[99].

T_C cells also play a pathogenic role in MS and EAE and models based on T_C cells exist as well. In these models, EAE is induced either by adoptive transfer of activated myelin-specific T_C cells or by generation of $CD8^+$ TCR transgenic mice[80,100]. EAE manifestation is different from T_H cell-mediated EAE, but resembles many features of MS[80,100].

Each of the animal models discussed above reproduces only part of the heterogeneous pathological features seen in MS, and to recapitulate the whole spectrum of the disease and understand its pathogenesis they need to be analyzed in combination[79,80].

2.4. Pathogenesis

While the triggering event in MS pathogenesis is not clear, it is widely accepted that an autoimmune T_H cell response against myelin components is central to this process[65,68]. Presumably, myelin-specific autoreactive T_H cells that escape deletion in the thymus, become activated in the periphery and cross the blood-cerebral

spinal fluid (CSF) or blood-brain barrier (BCB and BBB, respectively) to be reactivated in the CNS by resident APC[65] (*Fig.6*).T cell reactivation triggers effector function that initiates recruitment of innate and adaptive immune cells[65] (*Fig.5*). This establishes an inflammatory environment, causing damage to CNS-resident cells, such as oligodendrocytes and neurons, leading to demyelination and axonal damage and ultimately to development of focal lesions and neurological symptoms[65] (*Fig.6*).

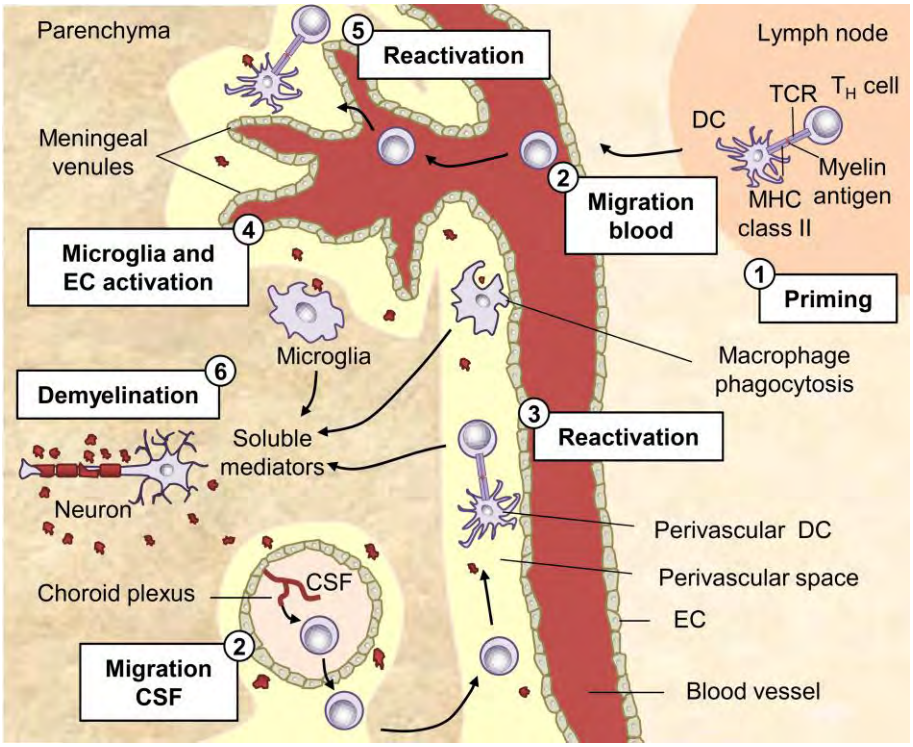

Figure 6. Peripheral activation and CNS invasion by T_H cells. T_H cells are primed in the periphery by DC presenting myelin epitopes (**1**). T_H cells cross the BCB barrier either in the choroid plexus or the meningeal venules (**2**); the T_H cells are reactivated within the subarachnoid space by DC expressing myelin epitopes (**3**). Reactivated T_H cells trigger activation of distal microglial cells and blood vessels (**4**). Activated T_H cells adhere to and cross the activated endothelium of the BBB, enter the perivascular space and are reactivated by perivascular DC (**5**). T_H cells enter the parenchyma and, together with activated Mø and microglial cells, secrete soluble mediators that cause demyelination (**6**). *Adapted from Goverman (2009) Nat. Rev. Immunol.*

2.4.1. Antigenic specificity and epitope spreading

What is the exact antigen against which the T_H cell response is triggered is a pressing question in MS and despite considerable effort no definitive answer has been reached yet. Several lines of evidence suggest that this antigen could be part of the myelin sheath. An indirect but consistent observation is that T_H cells from individuals with MS are restricted to MS-associated HLA class II molecules and reactive with several myelin epitopes, such as MBP, PLP and MOG, and the avidity of these cells is higher for those demonstrated to be able to induce EAE[68]. Even though the antigenic specificity does not differ from healthy individuals, the frequency of primed T cells specific for encephalitogenic epitopes is increased in MS individuals[68]. In some patients, a transient increase in MBP-reactive T cells correlates with higher disease activity[68]. In addition, MS-associated HLA class II molecules are overrepresented in individuals with MS and a small but substantial increase in the frequency of high-avidity T_H cell lines recognizing myelin epitopes that are weak HLA binders was reported in MS patients[68].

Although this data supports the hypothesis that the antigen in MS is a myelin component, it is possible that the relevant antigen is a pathogen-derived epitope or even another constituent of the CNS. T_H cell responses against several other non-myelin proteins occur in MS individuals including: 2',3'-cyclic nucleotide 3' phosphodiesterase, α-B-crystallin, S100β protein and transaldolase-H[68]. Lipid components are also candidate antigens in MS, as T cell responses against gangliosides are found increased in individuals with PP-MS[68]. However, it is not known which is the disease-inducing antigen and which antigens are the result of epitope spreading, reported in individuals with MS[101]. In EAE, epitope spreading has been extensively characterized and is the major functional cause of disease progression[68,102]. It starts in the inflamed CNS[103] where DC are the main APC responsible for epitope spreading[103-105]. DC are found in MS lesions[106] and individuals with MS, have higher levels of blood-derived myeloid DC expressing the activation makers CD40 and CD80, and the pro-inflammatory cytokines IL-12 and TNF[107]. In EAE, DC present in the CNS have been shown to activate naïve myelin-

specific T cells that were recruited to the inflamed tissue and to contribute to their differentiation into T_H17 cells[108].

2.4.2. Break of peripheral tolerance and CNS invasion

The ability of myelin-specific T_H cells to infiltrate the CNS requires these cells to be previously activated in the periphery. CNS antigens are continuously transported to cervical LN via CSF drainage[65]. In transgenic mice expressing a specific TCR for either MBP or PLP and developing EAE spontaneously, the first MBP and PLP-specific T cell responses occur in the CNS-draining cervical LN[109,110] (*Fig.6*). This suggests that myelin-specific antigens are presented here by APC and that peripheral tolerance mechanisms must operate in CNS-draining cervical LN to prevent activation of naïve myelin-specific T_H cells[65]. However, under some conditions, not clearly understood at the moment, presentation of myelin epitopes leads to activation of myelin-specific T_H cells[65] (*Fig.6*). The reason why under specific circumstances, APC present myelin antigens in an immunogenic manner is not clear, but could involve molecular mimicry and/or bystander activation, such as provided by a concomitant infection, triggering a pathologic autoimmune response (*see section 2.2.2*).

Once activated in CNS-draining cervical LN, myelin-specific T_H cells access to the CNS is limited by two barriers: the BCB and the BBB[65] (*Fig.7*). The BCB is constituted by epithelial cells and surrounds the choroid plexus (where CSF is produced) and the meningeal venules, while the BBB is composed by endothelial cells (EC) and surrounds parenchymal venules[65] (*Fig.7*). The cells forming both barriers are united by tight junctions and limit CNS access to memory T cells that play an immunosurveillance role in the CNS[65]. This is thought to occur mainly by cells that cross the BCB into the subarachnoid space, as epithelial cells of the BCB are the only ones that express constitutively the adhesion molecules necessary for transmigration of activated T cells[111]. In the context of EAE, leukocytes, and in particular T cells, are thought to cross the BCB barrier first (*Fig.6*). CC-chemokine ligand 20 (*Ccl20*) is constitutively expressed by epithelial cells of the choroid plexus, and its receptor CC-chemokine receptor 6 (*Ccr6*), expressed on T_H17 promotes the

migration of these cell into the CNS[112]. Both DC and Mø are present in the CSF of healthy individuals[113] and DC are sufficient to reactivate primed T cells in the CNS and induce EAE[114]. Mø depletion inhibits EAE, supporting a role for these cells in disease initiation[115]. Nevertheless, it has not been excluded by this study whether the effect is also mediated by DC, as the method used for Mø depletion is based on the phagocytic capacity of cells and thus, also eliminates DC[116].

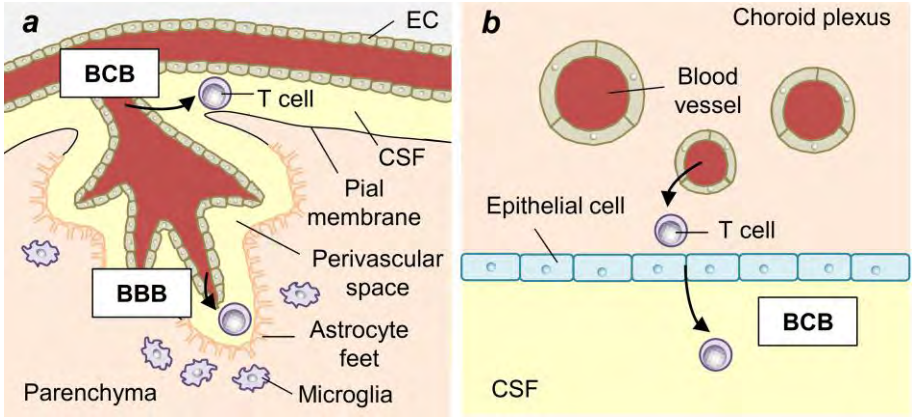

Figure 7. Routes of entry of activated T cells in the CNS. (***a***) Activated T cells that cross the BCB, through EC of meningeal venules, enter the subarachnoid space, between the arachnoid and pial membranes where CSF circulates[65]. They can also enter the subarachnoid space by migrating from blood vessels into the stroma of the choroid plexus and then crossing the BCB (***b***). Alternatively, they can cross the EC layer joined by tight junctions at the BBB of post-capillary venules that penetrate the brain parenchyma, entering the perivascular space. This space is the region between the basement membrane connected to the blood vessel EC and the glial limitans, which is composed of astrocyte feet and microglial cells[65] (***a***). *Adapted from Goverman (2009) Nat. Rev. Immunol.*

T cell reactivation in the CNS leads to proliferation and secretion of pro-inflammatory cytokines. These activate EC of the BBB that induce the expression of chemokines and adhesion molecules facilitating perivascular infiltration[65] (*Fig.6*). The transition of T cells from the perivascular space into the CNS parenchyma is dependent on chemokines produced by parenchyma cells in the CNS[65]. The degree of ensuing CNS inflammation is not determined by the ability of T cells to invade the CNS parenchyma, but by the relative strength of T cell reactivation by resident APC in the CNS[65].

2.4.3. Cellular and soluble effectors of CNS damage

Effector cells and molecules in MS are directly responsible for the damage imposed to parenchyma cells in the CNS. Cellular effectors include innate immune cells, i.e. Mø/microglia, and adaptive immune cells, such as T_H1 and T_H17, T_C and B cells. Soluble mediators comprise antibodies, complement activation components and ROS.

2.4.3.1. Innate immune cellular effectors

Mø/microglia contribute to autoimmune neuroinflammation by guiding and activating T cells and establishing an inflammatory milieu in the CNS[117] (*Fig.8*). They become potent APC and secrete pro-inflammatory mediators, such as IL-1, IL-6, TNF, nitric oxide (NO) and ROS[117] (*see section 2.4.3.3*). The production of ROS as a result of microglia activation can promote neuronal damage and reactive microgliosis (i.e. response of microglia to neural tissue damage) at sites of lesion[117]. Other innate immune cells such as γδ T cells, NK and mast cells have also been implicated in autoimmune neuroinflammation. However, their role is still not clear, with some studies suggesting a pathogenic and others a protective role in EAE[118].

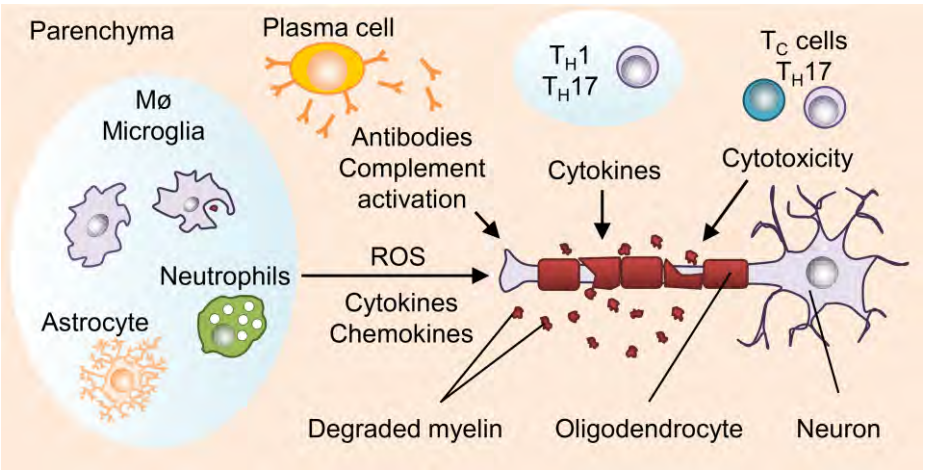

Figure 8. Effector mechanisms of CNS damage. T_H17 cells infiltrate the CNS and induce the secretion of chemokines that recruit Mø, neutrophils and more T cells (T_H17, T_H1 and T_C cells). T_H17 can also kill neurons by direct contact. All these cells produce cytokines that activate infiltrating Mø, microglia, neutrophils and astrocytes, leading to the production of pro-inflammatory mediators (i.e. ROS, chemokines and cytokines) and sustaining neuroinflammation. ROS can damage myelin via oxidation of lipids. Plasma cells secrete anti-myelin antibodies that opsonize myelin for phagocytosis by Mø. T_C cells can kill neurons and oligodendrocytes via several cell-cell contact mechanisms.

2.4.3.2. Adaptive immune cellular effectors

The cytokine profile of pathogenic T_H cells in MS is still an area of controversy. In EAE, T_H1, T_H17 and T_H9 cells can cause autoimmune neuroinflammation[65,86]. Among these, T_H1 and T_H17 cells seem to play a predominant role. However, the signature cytokines of these T cell lineages, i.e. IFN-γ and IL-17 are not the critical factors that determine T cell encephalogenicity as revealed by genetic ablation of either cytokine in mice[119,120]. Instead GM-CSF produced by T_H cells is necessary for T_H cell encephalogenicity[121]. In addition, IL-23, T-bet and STAT-4 also appear to be critical[122-125]. It is unclear, however, whether these molecules contribute to a common, yet undefined pathway, or act in a synergistic manner to promote encephalitogenicity. Nevertheless, both T_H1 and T_H17 cells seem to contribute to autoimmune neuroinflammation, since disease develops in the absence of molecules essential for the induction of each lineage, as revealed for *Il12p35*[100], *Ifng*[119], *Ifngr*[126], *Il17a* and *Il17f* [120]. Supporting a role for these cells in EAE and MS is the following set of observations: 1) Adoptive transfer of T_H1 or T_H17 cells is sufficient to induce EAE[87,88]; 2) increased levels of IFN-γ and IL-17 are detected in the CNS of animals undergoing EAE and in MS lesions[127-130]; 3) mice deficient in *Il23p19*, a cytokine required for T_H17 function are resistant to EAE[131] and 4) IFN-γ administration exacerbates MS[132], while IFN-γ neutralization ameliorated the pathology[133].

T_H17 cells are thought to constitute the first wave of cells to enter the CNS via a mechanism involving Ccr6, expressed on T_H17 cells, and its ligand Ccl20, expressed on epithelial cells of the choroid plexus[112]. Once in the CNS, T_H17 cells promote further recruitment of T_H1 and T_H17 cells, monocytes and neutrophils in a Ccr6-Ccl20 independent manner[112]. In the CNS of mice undergoing EAE, IL-17A-expressing cells convert into IFN-γ-producers and become the major source of this cytokine[134]. This conversion is dependent on IL-23[134], possibly explaining why *Il23p19*-deficient mice are resistant[122], while *Il12p35* remain susceptible to EAE[100]. This could also explain why IL-23 is required to initiate disease but not in the effector phase[135].

The exact role of T_H1 and T_H17 cells in the development of MS lesions is not clearly established but it is thought to rely on the differential pattern of chemokine and cytokine production[89,136] (*Fig.8*). T_H1 cells induce the expression of CXCL9, CXCL10 and CXCL11 that attract preferentially inflammatory monocytes, while T_H17 cells recruit preferentially neutrophils via CXCL1 and CXCL2[136]. IFN-γ produced by T_H1 cells induces the expression of MHC class II and adhesion molecules on APC, promoting T_H cell reactivation in the CNS. IFN-γ also activates microglia/Mø and astrocytes to secrete pro-inflammatory mediators[136] (*Fig.8*). The mechanisms by which T_H1 cells cause tissue damage *in vivo* are not fully elucidated. T_H1 cells are capable of inducing programmed cell death of antigen-presenting astrocytes via a mechanism that relies on MHC class II and IFN-γ[89]. Human oligodendrocytes are sensitive to IFN-γ-induced apoptosis and IFN-γ expression co-localizes with apoptotic oligodendrocytes in MS lesions[137].

T_H17 cells acquire cytotoxic potential *in vitro* only after converting to IFN-γ-producing cells[89]. T_H17 cells can induce severe, localized, and partially reversible fluctuations in neuronal intracellular calcium concentration as an early sign of neuronal damage[138] (*Fig.8*).

Evidence for a pathogenic role for T_C cells in the context of autoimmune neuroinflammation[139] includes the observation that under conditions where T_H cell depletion fails to improve MS, combined depletion of T_H and T_C cells inhibits disease progression[140]. A higher frequency of T_C cells recognizing myelin proteins is present in individuals with MS compared to healthy controls[141] and T cell receptor analysis of T_C cells from MS lesions reveals clonal expansion, which is consistent with recognition of cognate antigen[142,143]. Further supporting this notion, EAE can be induced by adoptive transfer of T_C cells[144,145]. Interestingly, EAE in *Cd8* knockout mice presents reduced mortality but increased relapse, suggesting that T_C cells might also play an immunoregulatory role in promoting periods of remission[146].

Although the pathogenicity of T_C cells is established, the effector mechanisms by which these cells contribute to lesion formation remain to be determined. T_C cells can exert cytotoxic effects in a MHC class I-restricted manner.

As such, T_C cells can kill neurons *in vitro* via cell contact-dependent mechanisms involving TNF receptor superfamily member 6 ligand (FasL), leukocyte function-associated antigen 1, and CD40[147], as well as oligodendrocytes by release of perforin[148] (*Fig.8*).

Self-reactive B cells are also important in the pathogenesis of MS. B cells and plasma cells are found in MS lesions[149] and B-cell depletion studies revealed a decrease in inflammatory activity in MS[150]. In EAE, B-cell deficient mice fail to develop EAE if immunized with the MOG protein[151].

2.4.3.3. Soluble effectors: antibodies, complement components and ROS

A pathogenic role for antibodies has been established by several lines of evidence. Up to 50% of the lesions in individuals with MS are characterized by abundant antibody deposition[69]. Clinical trials using plasmapheresis, a technique that removes soluble components from the serum, can reverse severe neurological deficits[152]. Antibodies against CNS components such as MOG, MBP peptides, α-B-crystallin and β amyloid[68], as well as against lipids of the myelin sheath, such as sulfatides, cerebrosides and phospholipids have all been found in the brain and/or CSF of individuals with MS[153]. In addition, in the CSF of MS patients there is oligoclonal distribution, i.e. only some clones are represented, suggesting there is antigen-driven clonal selection[154].

Antibodies can promote demyelination by opsonization of myelin for phagocytosis and via activation of the complement cascade (*Fig.8*). In MS lesions where antibodies are found, deposition of terminal components of the complement cascade are also present[155]. In addition, mice deficient for early components of the complement cascade develop attenuated EAE, suggesting that complement-mediated opsonization is involved in demyelination[156].

ROS are products of normal cellular metabolism that play a physiological role in numerous cellular processes[157]. They are generated by the mitochondrial respiratory chain during aerobic respiration and include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$) and organic peroxides, as products of the biological reduction of molecular oxygen[158,159]. ROS are also

produced by Mø[160] and neutrophils[161]. Normally, ROS are quenched by antioxidant mechanisms of the cell that comprise enzymes, such as catalase or superoxide dismutases (*Table 1*), and soluble molecules, such as glutathione and thioredoxin[157,162] (*Table 1*). Whenever the rate of production of free radicals, exceeds the capacity of the protective antioxidant mechanisms, oxidative stress ensues. This can lead to damage of lipids, proteins and DNA[157,163], causing mutations and epigenetic alterations.

Individuals with MS have lower concentrations of antioxidants in the serum, compared to healthy individuals, and MS lesions show reduced levels of antioxidants and several markers of oxidative damage[163]. This is suggestive that antioxidant depletion results from continued exposure to ROS. Oxidative stress is thought to contribute to the formation and persistence of MS lesions. In the brain parenchyma, ROS contribute to tissue damage mediating myelin phagocytosis, oligodendrocyte damage and axonal injury[164,165] (*Fig.8*). Furthermore, dietary intake of exogenous antioxidants, including flavonoids and α-lipoic acid, reduces the clinical signs of EAE[166,167]. This supports the notion of a role for oxidative stress in the effector damage during EAE and MS.

Adaptive responses to oxidative stress can counter oxidative damage, such as occurs in the CNS during MS and EAE. These rely on the activation of transcription factors that include nuclear factor erythroid (NF-E) 2-related factor 2 (Nrf2)[157].

3. The Nrf2 cytoprotective pathway

Nrf2 is an evolutionarily conserved basic leucine-zipper (bZip) transcription factor expressed by worms, insects, fish, birds and mammals but not by plants or fungi. Activation of Nrf2 mediates cellular adaptive responses to oxidative stress[162]. Nrf2 contains a conserved Cap 'n' Collar (CNC) domain (*Fig.9*) that defines a family of transcription factors that, in mice and humans, includes Nrf1, Nrf2, and Nrf3[168,169]. Broad-Complex, Tramtrack, and Bric-a-brac (BTB) and CNC homology (Bach) 1 and Bach2 are more distantly related family members containing an additional BTB domain, acting as transcriptional repressors[168,169]. A naturally-occurring truncated

isoform of Nrf1 as well as a caspase-cleaved form of Nrf2 may also function as transcriptional repressors[168,169].

Nrf2 is activated by changes in the redox status of the cell in response to oxidants (remove electrons from another reactant) and electrophiles (accept a pair of electrons to make a new covalent bond). Nrf2 activation restores homeostasis by up-regulating a diverse set of cytoprotective genes (*see section 3.2*)[162,168]. Thus, Nrf2 influences the sensitivity to physiologic and pathologic processes affected by oxidative and electrophilic stress, such as those imposed by exposure to environmental toxicants and inflammation (*see section 3.3*)[162]. This is illustrated by increased sensitivity of *Nrf2*-deficient (*Nrf2*$^{-/-}$) mice to diverse oxidative insults. When exposed to xenobiotic electrophiles, or chemicals that generate oxidative stress, *Nrf2*$^{-/-}$ mice display higher amount of DNA, lipid and protein oxidation, tissue damage and inflammation, as compared to wild-type animals[169]. *Nrf2*$^{-/-}$ mice are also more sensitive to a number of inflammatory diseases, such as sepsis, systemic lupus erythrematosus, pulmonary diseases, colitis, rheumatoid arthritis and EAE[162,169,170]. Compounds that activate the Nrf2 pathway, e.g. curcumin and sulforaphane, act as therapeutic agents in several experimental models of toxicity, neurodegeneration and inflammation-driven diseases (*see section 3.3*)[162,169]. Clinical studies have also determined the therapeutic value of pharmacologic induction of the NRF2 pathway in the treatment of inflammatory lung diseases, neurodegeneration and preeclampsia[169].

In humans, analysis of disease-associated DNA polymorphisms also supports a protective role for NRF2[171]. DNA polymorphisms that reduce NRF2 expression are associated with skin vitiligo, chronic gastritis, peptic ulcer, ulcerative colitis and adult respiratory distress syndrome[169]. These observations suggest that individuals bearing these polymorphisms have a reduced antioxidant response and are at increased risk for diseases related to oxidative stress[169].

Nrf2 binds to the antioxidant response element (ARE) with the consensus sequence TGACnnnGC[168], present in enhancer elements in the promoter regions of cytoprotective genes[172]. Nrf2 forms heterodimers with small musculo-aponeurotic fibrosarcoma oncogene (Maf) proteins (MafF, MafG and MafK) and binds to the

ARE, recruiting the general transcriptional machinery and inducing the expression of ARE-driven cytoprotective genes (*see section 3.2*)[162,172].

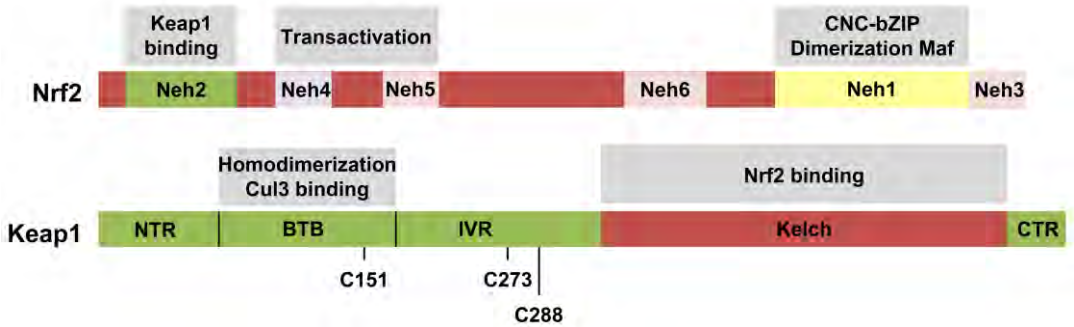

Figure 9. Structural domains of Nrf2 and its inhibitor Keap1. In the Nrf2 protein are shown the relative positions of the 6 functional domains, Nrf2-ECH homology (Neh) 1-6[168,169]. Neh1 contains the bZip DNA binding and heterodimerization domain through which Nrf2 binds its transcriptional partners, including small Maf proteins[168,169]. Nrf2 is negatively regulated by Keap1 that interacts with the Neh2 domain[168,169]. The Neh3 domain binds to chromodomain helicase DNA binding protein 6 that functions as a transcriptional co-activator[168,169]. Neh4 and Neh5 act synergistically to bind the transcriptional co-activator CREB binding protein (CBP)[168,169]. Finally, the Neh6 domain controls the Keap1-independent negative regulation of Nrf2[168,169]. In the Keap1 protein are shown the N-terminal region (NTR), the BTB domain, the intervening region (IVR), the Kelch domain and the C-terminal region (CTR). The BTB is the domain through which Keap1 homodimerizes and the Kelch domain through which it binds Nrf2[168]. In the BTB and IVR domains are present reactive cysteines C151, C273 and C288 through which Keap1 senses redox changes[168,169].

3.1. Regulation of the Nrf2 pathway

Nrf2 is regulated mainly at the post-transcriptional level by the Kelch-like ECH associated protein (Keap1)[168,169], a cysteine-rich protein, containing 25 or 27 cysteine residues in mice and humans (*Fig.9*). Ten of these residues are predicted to be reactive with oxidants or electrophiles, breaking the disulfide bond between two adjacent cysteines, which allows Keap1 to sense redox changes and triggers the activation of the Nrf2 pathway[168,169] (*Fig.9*). Nrf2 also contains four evolutionarily conserved cysteine residues, suggesting that it could react directly with oxidants or electrophiles, but the physiological relevance of this is still an open question[168].

Under homeostasis, Nrf2 is sequestered in the cytoplasm by Keap1, targeting Nrf2 for proteolysis by the 26S proteasome (*Fig.10a*). Under oxidative stress, this association is modified, leading to diminished rates of proteolysis of Nrf2 and enhanced nuclear accumulation. Nrf2 heterodimerizes with small Maf proteins and drives the transcription of ARE-regulated genes (*Fig.10b*).

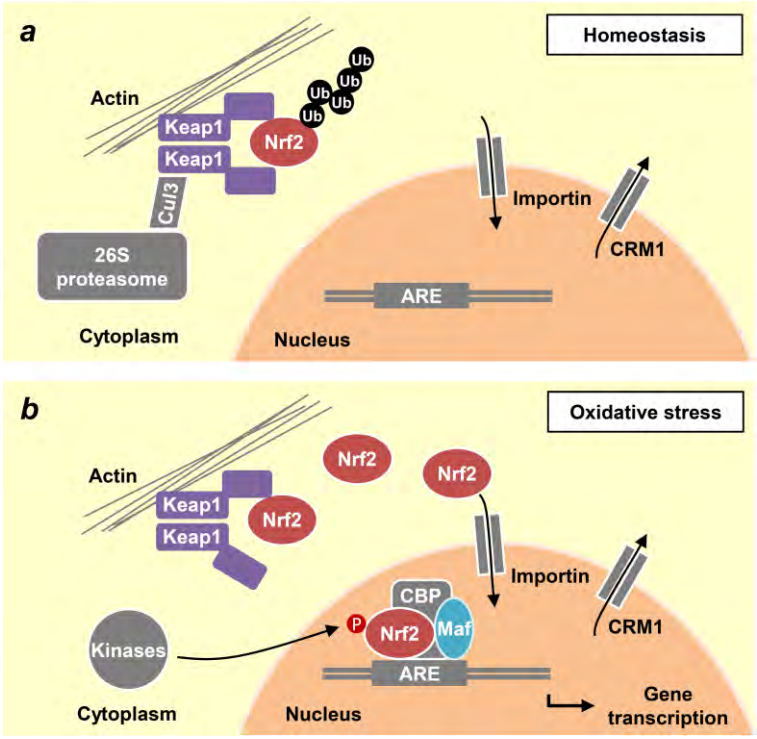

Figure 10. Nrf2 regulation by Keap1. (***a***) In homeostasis, Keap1 is tethered to the actin cytoskeleton and localizes in the cytoplasm[168,169]. It binds Cul3, which forms a core E3 ubiquitin ligase complex through association with Ring-box1 protein (Rbx1)[168,169]. Keap1 negatively regulates Nrf2 activity via cytoplasmic sequestration and targeting of Nrf2 for proteasomal degradation, acting as an E3 ubiquitin ligase substrate adaptor for the Cul3-Rbx1 complex[168,169]. Nrf2-Keap1 interaction is thought to occur via the "hinge and latch" mechanism, with a high-affinity and a low-affinity motif, within the Neh2 domain of Nrf2 interacting with a separate Kelch repeat domain in the Keap1 homodimer. Both motifs are required for the Nrf2 repression by Keap1. (***b***) Oxidants and electrophiles react with cysteines in Keap1, leading to a conformational change and release of the weaker interaction with the low affinity motif of Nrf2. Failure of Nrf2 to interact simultaneously with both Kelch repeat domains enables it to "escape" ubiquitination by Cul3-Rbx1, reducing its degradation rate[168,169]. This impairs newly transcribed Nrf2 from binding Keap1, allowing for Nrf2 nuclear translocation and transcriptional regulation. Phosphorylation of Nrf2 by several kinases also affects its nuclear accumulation. Nuclear export is mediated by the exportin chromosome region maintenance (CRM1).

Some inducers of the Nrf2 pathway can stabilize Nrf2 by dissociating the Keap1-Cul3 complex, thus leading to the inhibition of Nrf2 ubiquitination. Other inducers of the pathway may target Keap1 for Cul3-mediated ubiquitination[168,169]. It

has also been suggested that the nuclear translocation of Nrf2 is redox regulated, fine-tuning the speed, magnitude and duration of the antioxidant response[169]. It is possible that these mechanisms are not mutually exclusive and just reflect inducer and/or cell type-specific regulatory mechanisms.

Although the main form of Nrf2 regulation relies on the modulation of Keap1-dependent Nrf2 degradation, there is also a mechanism of Keap1-independent Nrf2 degradation. The Neh6 domain may contain a redox-insensitive degron (a specific sequence of amino acids that directs the starting place of degradation)[168,169]. Glycogen synthase kinase (GSK) 3β and the β-transducin repeat containing E3 ubiquitin protein ligase regulate the activity of the Neh6 degron in a Keap1-independent manner[168,169]. In addition, Nrf2 is also regulated at the transcriptional and translational levels. There is an ARE in the Nrf2 promoter, suggesting Nrf2 regulates its own transcription in a positive feedback loop[168,169]. At the translational level, Nrf2 mRNA contains a redox-sensitive internal ribosomal entry site in the 5'-UTR, increasing Nrf2 translation upon redox imbalance[168,169]. These levels of regulation may function in concert with increased Nrf2 stability to provide a robust long-term response to inducers.

The Nrf2-Keap1 signal transduction pathway interacts at multiple levels with other signal transduction pathways. These include phosphorylation of Nrf2, by a number of protein kinases that regulate its activity, e.g. protein kinase C, MAPK, GSK3β, tyrosine kinase Fyn, protein kinase-like endoplasmic reticulum kinase and casein kinase II[168,169] (*Fig.10a*). Nrf2 can also compete for ARE binding with MafK and with transcription factors, such as Nrf1, Nrf3, Bach1, activating transcription factor (ATF)-1, ATF-4, JunD, c-Jun, c-Fos and Fra1[169]. Nrf2 binding to ATF-3, proliferator-activated receptor (PPAR)-γ, RORα, estrogen-related receptor β, estrogen receptor α, the chromatin remodeling factor brahma-related gene 1 and the transcriptional co-repressor silencing mediator of retinoic acid and thyroid hormone receptor also modulates Nrf2-driven gene expression[169]. Finally, there is cross-regulation with the AhR, NF-κB, p53 and Notch pathways at the transcriptional level[173].

3.2. Transcriptional regulation of cytoprotective genes

Nrf2 has an estimate of 600 target genes arguing for the central role of this transcription factor in the cytoprotective response to oxidative stress (*Table 1*). These genes encode proteins: acting as direct antioxidants, inactivating oxidants, increasing the levels of glutathione synthesis and regeneration, stimulating NADPH synthesis, enhancing toxin export through the multidrug-response transporters, enhancing recognition, repair and removal of damaged proteins, elevating nucleotide excision repair, regulating the expression of other transcription factors, receptors and molecular chaperones and dampening inflammation[162,168] (*Table 1*).

Name	Gene	Function
Glutathione S- transferases	*Gstt1*	Detoxify electrophiles by the conjugation with glutathione.
Metallothioneins	*Mt1* *Mt2*	Bind heavy metals preventing heavy metal-mediated oxidative stress.
Thioredoxins	*Trn1*	Quench reactive oxygen species undergoing reversible oxidation.
Peroxiredoxins	*Prdx1*	Degrade enzymatically hydrogen and organic peroxides and peroxynitrite.
Catalase	*Cat*	Catalyses the conversion of hydrogen peroxide into H_2O and O_2.
Superoxide dismutases	*Sod2*	Catalise the dismutation of superoxide anion into H_2O_2 and O_2.
NAD(P)H:quinone oxidoreductases	*Nqo1* *Nqo2*	Catalyze the two-electron reductions of quinones and derivatives.
γ-glutamyl cysteine ligase	*Gclm* *Gclc*	Catalyzes the rate-limiting step in the biosynthesis of glutathione.
Glutathione peroxidases	*Gpx1*	Detoxify cellular hydrogen and organic peroxides by oxidizing glutathione.
Heme oxygenase-1	*Hmox1*	Catabolizes heme into CO, biliverdin and labile Fe.
Ferritin H chain	*Fth*	Sequesters and oxidizes labile Fe stopping it from being a Fenton reactor.
CD36 antigen	*Cd36*	Cell surface receptor of several ligands: i.e. oxidized lipoproteins.

Table 1. **Nrf2-regulated genes and their functions.** Examples or Nrf2-regulated genes that are involved in: 1) providing direct antioxidants (i.e. *Mt* and *Trn*); 2) encoding enzymes that directly inactivate oxidants, (e.g. *Gstt1, Prdx*, *Cat*, *Sod*, *Hmox1*, *Fth* and *Nqo*), 3) increasing the levels of glutathione synthesis and regeneration, (i.e. *Gpx1*, *Gsr*, *Gclm* and *Gclc*) and 4) dampening inflammation, i.e. *Cd36*[162,168].

The heme oxygenase-1 (*Hmox1*) gene, encoding the heme-catabolizing enzyme HO-1 (*see section 3.2.1*), is a Nrf2-regulated gene that acts in a salutary

manner in a number of immune-mediated inflammatory diseases, including autoimmune neuroinflammation[174-176] (*see section 3.3.1*). This suggests that the salutary effects of Nrf2 exerted in autoimmune neuroinflammation, and presumably in other immune-mediated inflammatory diseases (*see section 3.3*), could be mediated, at least in part, via the expression of HO-1.

HO-1 is regulated mainly at the transcriptional level, with its rate of transcription being induced by a variety of signal transduction pathways that culminate in the activation of Nrf2 (*Fig.11*), as well as other transcription factors, such as NF-κB, AP-1 and heat shock factor[177].

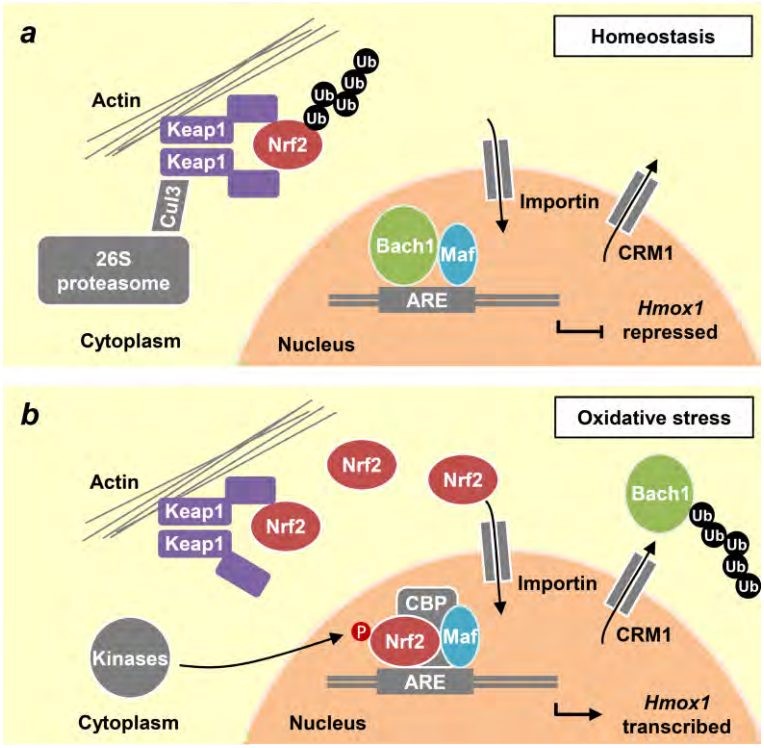

Figure 11. Regulation of *Hmox1* transcription by the Nrf2 pathway. (***a***) In homeostasis Bach1 in heterodimer with small Maf proteins binds to the ARE in the enhancers of the *Hmox1* promoter repressing its transcription. (***b***) Upon oxidative stress and/or an increase in heme levels the DNA-binding activity of Bach1 is inhibited and its nuclear export via CRM1 and proteolysis promoted. This allows Nrf2-small Maf heterodimers to bind the ARE and drive *Hmox1* transcription.

Several *cis*-acting (acting on the same chromosome) regulatory elements have been identified in the *Hmox1* promoter, including upstream enhancer regions, E1 and E2, which play a major functional role in the redox-dependent induction of HO-1, as they contain several ARE[178].

Nrf2 binding to these ARE, in association with small Maf proteins, leading to transcription of *Hmox1*, is dependent on release of the transcriptional repressor Bach1 from the ARE[179] (*Fig.11*). Under homeostasis, HO-1 expression is repressed by Bach1-small Maf protein heterodimers (*Fig.11a*), but upon oxidative stress, Bach1-mediated repression of HO-1 is relieved (*Fig.11b*). Bach1 can sense ROS and heme, which inhibits its binding to the ARE and promotes nuclear export and degradation by the 26S proteasome[178] (*Fig.11b*). Other levels of regulation of HO-1 have also been described, including post-transcriptional regulation by mRNA stabilization and post-translational regulation by cleavage of HO-1[180].

3.2.1. HO-1 and the products of heme catabolism

HO-1 is the enzyme responsible for the first and rate-limiting enzymatic step in the catabolism of heme (Fe protoporphyrin IX), generating equimolar amounts of biliverdin, carbon monoxide (CO) and labile Fe[181] (*Fig.12*). HO-1 is one of two evolutionarily conserved isozymes, HO-1 and HO-2, encoded in mice and humans by the *Hmox1*/*HMOX1* and *Hmox2*/*HMOX2* genes, respectively. *Hmox* genes are evolutionarily conserved and ubiquitously expressed in most living organisms including bacteria, algae, plants, insects and mammals, suggesting that the need for heme catabolism appeared early in evolution[182]. HO-2 is a non-inducible enzyme[183], while HO-1 is inducible by a wide variety of oxidative stress-related stimuli[184] including hypoxia, shear stress, ROS, heavy metals, cytokines, e.g. TNF, IL-1β, TLR ligands, such as LPS, and its substrate, heme[177,178].

HO-1 provides cellular protection against oxidative stress, preventing programmed cell death, and thus providing beneficial effects in many inflammatory conditions[185]. In support of this notion are the observations that cells lacking HO-1 expression are highly sensitive to oxidative stress, which leads to exacerbated cellular toxicity[186]. In addition, induction of HO-1 expression or HO-1 over-

expression prevents oxidative stress-mediated toxicity in a variety of cell types[187,188].

Mice with a disruption of the *Hmox1* gene (*Hmox1*$^{-/-}$) die *in utero* at high frequency, presumably due to lack of protection against the oxidative stress generated during embryonic development[189-191]. Moreover, the embryos that survive to term display a chronic inflammatory condition that progresses with age and can cause premature death[189]. *Hmox1*$^{-/-}$ mice show increased sensitivity to a number of inflammatory pathologies (*see section 3.3.1*), in which oxidative injury is a common pathologic denominator. These include but are not limited to: graft rejection[192], atherosclerosis[193], ischemia-reperfusion injury[194], septic shock[195,196] and malaria[197,198]. In addition, pharmacological induction of HO-1 or administration of the end products of its activity can exert therapeutic effects in a variety of inflammatory diseases[176].

In humans, *HMOX1* deficiency is extremely rare and leads to the development of an inflammatory syndrome that can cause premature death[199]. This is characterized by massive EC damage, growth failure, anemia, tissue Fe deposition, lymphadenopathy, leukocytosis and increased sensitivity to oxidant injury[199]. In addition, the human *HMOX1* promoter has a (GT)n microsatellite polymorphism that alters the levels of HO-1 expression and has been associated with the incidence and/or progression of a variety of diseases[200]. Lower numbers of GT repeats within this polymorphic sequence have been associated with higher inducibility of HO-1 expression in response to stress stimuli and individuals with this allele seem to be less likely to develop a series of pathologies than individuals with a higher number of (GT)n repeats[200]. These pathologies overlap broadly with those in which the outcome is exacerbated in mice that lack HO-1 or in wild type mice, in which HO-1 activity is inhibited pharmacologically[176,200]. This illustrates further the crucial role of HO-1 in the prevention of oxidative damage associated with inflammation (*see section 3.3.1*).

Heme, the substrate of HO activity, consists of a protoporphyrin IX ring with a central Fe ion. It exists as a prosthetic group of hemoproteins, an evolutionarily conserved strategy that allows to incorporate Fe into the tertiary structure of

proteins[180]. It is an abundant compound in mammals and plays a physiological role for oxygen and mitochondrial electron transport as an essential prosthetic group of hemoglobin, myoglobin and cytochromes[180]. However, when released from the heme pockets of hemoproteins, heme can be highly toxic as it may react with H_2O_2 to generate $OH^{\cdot}$ via the Fenton chemistry, leading to oxidative stress[201,202]. Due to the pro-oxidant properties of non-protein bound heme, the enzymatic synthesis and degradation of this molecule must be tightly controlled[180].

Heme degradation by HO generates biliverdin, which is converted into bilirubin by biliverdin reductase[203] (*Fig.12*). Bilirubin is a lipophilic tetrapyrrole, abundant in blood plasma and bile that exists uniquely in mammals and can be toxic at high concentrations[204]. However, it also appears to be one of the most abundant endogenous antioxidants in mammals and accounts for the major antioxidant activity in human serum[205].

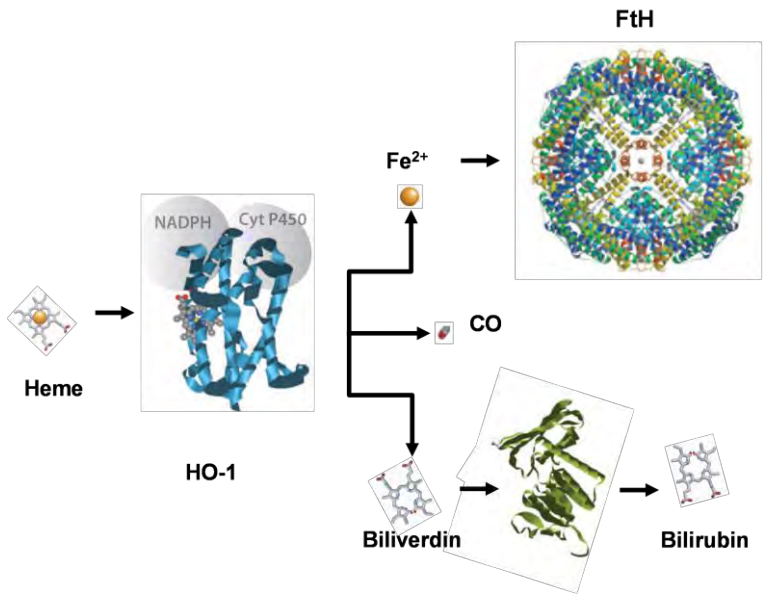

Figure 12. Heme catabolism by HO-1. Heme catabolism by HO-1 involves cleavage of the protoporphyrin IX ring, generating equimolar amounts of biliverdin, CO and labile Fe. The Fe^{2+} released by this reaction is then stored by FtH. Biliverdin is converted by biliverdin reductase into the antioxidant bilirubin . All three end products of heme catabolism, that is, biliverdin/bilirubin, CO and Fe/FtH, are cytoprotective. *Adapted from Gozzelino et al. (2010) Annu. Rev. Pharmacol. Toxicol.*

Another product of heme catabolism by HO is CO (*Fig.12*), a gaseous transmitter that modulates cellular signal transduction[206]. CO binds to Fe atoms in heme and/or sulfur clusters contained in a variety of proteins, e.g. guanylyl cyclase, cytochromes, peroxidases, catalase, nitric oxide synthases and phosphatases[207]. Presumably, when this occurs, the conformational structure of these proteins is modified in a manner that modulates their biological activity[208].

Heme degradation by HO unlocks a third product, labile Fe from the protoporphyrin IX ring (*Fig.12*). Labile Fe can be deleterious as it catalyzes the generation of ROS through the Fenton chemistry, promoting oxidative stress[209]. Labile Fe up-regulates the expression of an Fe-transporter pump that removes it from cells[210]. In addition, labile Fe induces the expression of the Fe-sequestering protein FtH[211] (*Fig.12*), which associates with ferritin L chain and forms a multimeric protein complex with exceedingly high Fe storage capacity[212]. FtH has ferroxidase activity that oxidizes Fe^{2+} into Fe^{3+}, which can limit the availability of labile Fe to promote the generation of ROS via the Fenton chemistry[212].

3.3. Regulation of immune-mediated inflammatory diseases by Nrf2

Nrf2 confers protection against inflammatory-mediated injury[162,169,170], as illustrated by the phenotype associated with deletion of the *Nrf2* allele in mice. Namely, *Nrf2*$^{-/-}$ mice display a shortened lifespan as compared to wild type (*Nrf2*$^{+/+}$) mice, presumably due to the development of a lupus-like autoimmune syndrome characterized by multi-organ damage, intravascular immune complex deposition, and the development of severe glomerulonephritis[213,214]. In addition, aged *Nrf2*$^{-/-}$ mice develop immune-mediated hemolytic anemia[215]. *Nrf2*$^{-/-}$ mice also develop over time (10 month after birth) widespread astrogliosis, along with vacuolar leukoencephalopathy[216].

Genetic disruption of the *Nrf2* allele increases susceptibility to a variety of experimental models of immune-mediated inflammatory diseases in mice, including septic shock, colitis, lung inflammation, leading to acute respiratory distress syndrome, asthma and emphysema[217-219] as well as diseases targeting the CNS, such as EAE[170,220,221] (*Table 2*). This is associated with increased expression of

several pro-inflammatory genes, suggesting that Nrf2 regulates the immune response during EAE. Furthermore, Dimethylfumarate, has also proved beneficial in EAE[221,222], and more importantly in MS[223,224], acting via the Nrf2 pathway[221,222].

Pathology	Pro-inflammatory mediators up-regulated
Septic shock	Cytokines (IL-1, TNF, IL-6, etc.), chemokines and adhesion molecules
Asthma	Cytokines (IL-4, IL-13)
Colitis	Cytokines (IL-1, IL-6, IL-12/23p40 and TNF)
Colitis	Cytokines (IL-1β and TNF) and adhesion molecules (ICAM-1)
EAE	Cytokines (*Ifng*, *Il1b*, *Tnf* and *Il12*) and chemokines (*Cxcl13* and *Cxcl9*)

Table 2. Genetic ablation of *Nrf2* leads to increased expression of pro-inflammatory mediators in diverse pathologies. Example of diseases where genetic ablation of *Nrf2* has been shown to exacerbate pathology and to be associated with increased expression of pro-inflammatory mediators.

The regulatory role of Nrf2 in these experimental models of disease is thought to be mediated by two mechanisms: 1) Activation of the cellular antioxidant and detoxifying machinery, providing cytoprotection and tissue damage control[162] and 2) Suppression of pro-inflammatory signaling pathways restraining the expression of pro-inflammatory mediators, including but not limited to cytokines, chemokines and cell adhesion molecules[170] (*Table 2*).

Nrf2 inhibits the expression of pro-inflammatory mediators, acting in distinct processes of the inflammatory response. This effect can be mediated by binding of Nrf2 to the promoter regions of Nrf2-dependent pro-inflammatory mediators, in a manner that would block their transcription, or more likely, given that Nrf2 is a transcriptional activator, relying on the interference of Nrf2 with pro-inflammatory signaling pathways. An effect of Nrf2 on a promoter has been suggested for VCAM-1 regulation, in a study where Nrf2 overexpression strongly inhibited VCAM-1 promoter activity and Keap1 reversed this effect[170]. Negative regulation of pro-inflammatory mediators by Nrf2 can also act indirectly through cytoprotection and tissue damage control, thus limiting release of DAMPs and sustained expression of pro-inflammatory mediators.

Expression of many inflammatory mediators, associated with the onset of inflammatory reactions, such as chemokines, cytokines, and cell-adhesion molecules, is regulated by the transcription factor nuclear factor-kappa B (NF-κB). NF-κB constitutes a family of transcription factors that contain a conserved Rel homology domain responsible for dimerization and DNA binding[173,225]. It can be subdivided into two groups based on the presence, (RelA/p65, RelB, and c-Rel), or absence (p100 and p105) of a transactivation domain. In homeostasis, NF-κB is located in the cytoplasm associated with its negative regulator IκB, which hides the nuclear-localization sequence and DNA-binding domain of NF-κB, preventing transcription[173]. Upon triggering of a signal transduction pathway that leads to NF-κB activation, IκB is phosphorylated by IκB kinases (IKK), allowing the release and nuclear translocation of NF-κB[173]. In addition, phosphorylation of NF-κB can modify its transcriptional activity, of which the best characterized example is RelA phosphorylation at S276 by protein kinase A[226].

Nrf2$^{-/-}$ mice have increased NF-κB activation when compared with wild type (Nrf2$^{+/+}$) mice, as demonstrated in the context of traumatic brain injury, LPS and TNF administration and infection by respiratory syncytial virus[173,225]. In keeping with this notion, pharmacological inducers of the Nrf2 pathway repress NF-κB activation[173,225]. The mechanism via which Nrf2 inhibits NF-κB activation is not clearly elucidated[173,225], but is thought to involve the inhibitor of NF-κB, IκB via a mechanism targeting the upstream IKK kinase activity responsible for IκB phosphorylation and leading to its degradation and activation of NF-κB[225]. As the NF-κB pathway can be activated by ROS, via IκB phosphorylation[227], it is plausible that the increased levels of ROS observed in *Nrf2*$^{-/-}$ mouse embryonic fibroblasts (MEF)[228] account for the heightened IκB phosphorylation. This would suggest that the inhibitory effect of Nrf2 on this pathway relies on the expression of antioxidant genes and maintenance of the redox-status of the cell by preventing ROS-mediated NF-κB activation. In keeping with this notion, it has been demonstrated that maintenance of the cellular levels of glutathione is important[217]. In addition, several

Nrf2-dependent genes that contribute to redox homeostasis have been shown to regulate NF-κB activity, including *Trn*, *Nqo1* and *Hmox1*. In particular, HO-1 modulates NF-κB activation via a mechanism that relies on its end products bilirubin and free Fe[229,230] and that does not involve IκB phosphorylation, targeting the phosphorylation of RelA, required for TNF-dependent NF-κB activation[230].

3.3.1. Regulation of immune-mediated inflammatory diseases by HO-1

Oxidative tissue damage associated with inflammation is thought to lead to the unlocking of heme from hemoproteins, generating non-protein bound heme, which has pro-oxidant and pro-inflammatory properties[231]. Thus, control of the non-protein bound heme levels by HO-1 should restrain inflammation and prevent further tissue damage, a notion consistent with the evidence that *Hmox1* deletion in mice and humans is characterized by neutrophil activation, infiltration and oxidative tissue damage[190,199,232]. In addition, the deleterious effects of non-protein bound heme in inflammatory disease models have been illustrated by our laboratory in malaria[197,198,233] and septic shock[196]. These studies show that enzymatic degradation of heme via HO-1 play a critical role in the prevention of programmed cell death, affording tissue damage control[234]. In addition, our laboratory has shown, in a septic shock model, that elevated non-protein bound heme levels, such as occur in *Hmox1*$^{-/-}$ mice, lead to tissue damage and release of DAMPs, i.e. HMGB1[196]. Thus, the cytoprotective effect of HO-1 appears to have a dual protective role, in that it sustains tissue/organ function, therefore inhibiting the release of DAMPs from dying cells[185]. This latter effect, presumably in combination with control of the pro-inflammatory non-protein bound heme levels, contributes to prevent unfettered inflammation and oxidative tissue damage.

HO-1 has also immunoregulatory effects as demonstrated by *Hmox1* deletion in mice that develop an inflammatory syndrome with several immune abnormalities, such as splenomegaly, increased baseline IgM levels and increased production of pro-inflammatory cytokines by LPS-stimulated splenocytes[235]. This

immune phenotype has been dissected and immunomodulatory effects suggested both for innate and adaptive immune cells.

Regulation of innate immune responses by HO-1 has been demonstrated in mice with myeloid-specific ablation of *Hmox1* in that HO-1 is required for IRF3 activation and the production of antiviral cytokines, e.g. IFN-β[175]. Regulation of IFN-β by HO-1 impacts T_H cell responses and EAE, as IFN-β blocks T_H17 differentiation[175]. This effect was suggested to be mediated by HO-1 expression in Mø, although the possible contribution of other myeloid cells has not been addressed. In Mø, HO-1 has also been shown to be up-regulated by LPS, leading to attenuation of the expression of pro-inflammatory genes, such as cytokines, i.e. TNF an IL-6[236]. However, it is not clear how relevant this effect is as *Hmox1*$^{-/-}$ or *Hmox1*$^{+/+}$ peritoneal Mø produce similar pro-inflammatory cytokine levels in response to LPS *in vitro*[185].

Concerning the effects of HO-1 in DC, mouse immature DC express negligible levels of HO-1, inducing its expression upon activation[185]. Intriguingly, rat and human immature DC express HO-1 constitutively, downregulating its expression upon activation[185]. In any case, pharmacologic induction of HO-1 inhibits mouse, rat, and human DC activation and immunogenicity[185]. Recently, a study has suggested that the protective effects of Dimethylfumarate, in EAE and presumably MS, are mediated in part by HO-1 via the regulation of cytokine production in DC[222].

The effects of HO-1 in DC might impact adaptive immune responses. In addition, several observations *in vitro* suggest that HO-1 also inhibits T cell activation, proliferation and/or effector function[185]. HO-1 has also been suggested to suppress T cell responses by regulating T_{REG} cell function[185]. However, analysis of T_{REG} cells from *Hmox1*$^{-/-}$ and wild type mice revealed that HO-1 does not play a role in T_{REG} cell development, peripheral maintenance and function in homeostasis[237].

3.3.1.1. Regulation of immune-mediated inflammatory diseases by the end products of heme-catabolism

Biliverdin, CO and labile Fe play regulatory roles in several inflammatory diseases, having both cytoprotective, and immunoregulatory effects. Bilirubin, generated by

biliverdin reductase, is a potent antioxidant[205]. As ROS can trigger programmed cell death, bilirubin has cytoprotective effects, preventing NO or H_2O_2-mediated programmed cell death[238]. In addition, biliverdin/bilirubin are also immunoregulatory, inhibiting mouse and human T_H cell activation *in vitro*[239]. *In vivo*, bilirubin suppresses T cell-driven inflammatory pathologies such as the rejection of transplanted organs[240] or autoimmune neuroinflammation[239]. The immunoregulatory effects of biliverdin/bilirubin are mediated via inhibition of the transcription factors, NF-AT and NF-κB, suppressing IL-2 production by T_H cells[240].

CO is anti-apoptotic in a variety of cell types[241]. The cytoprotective effect of CO is mediated by a mechanism that prevents heme release from hemoproteins, e.g. hemoglobin, thus blocking its pro-oxidant effects[436]. In addition, CO interferes with the MAPK signaling pathway preventing apoptosis, as illustrated originally in Mø[236] and thereafter in EC[241,242]. Regulation of the p38 MAPK signaling pathway by CO inhibits TNF production by Mø[236] via a mechanism involving the production of mitochondrial ROS[243]. When exposed to CO, Mø shift the production of the pro-inflammatory TNF to that of IL-10, a potent anti-inflammatory cytokine[236]. CO also induces production of another potent anti-inflammatory cytokine, TGF-β by a pathway that involves stabilization of hypoxia-inducible factor 1α, again via mitochondrial-generated ROS[244]. Production of mitochondrial-generated ROS in response to CO is also involved in the up-regulation of PPR-γ, which exerts anti-inflammatory effects in Mø[245]. Other effects of CO in Mø include the interference with the molecular machinery involved in TLR signaling by two distinct mechanisms. First, CO enhances calveolin-1 binding to TLR4, inhibiting downstream signaling and production of pro-inflammatory cytokines[246]. Second, CO inhibits the activity of NADPH oxidase, reducing ROS formation, upon TLR ligation, consequently inhibiting TLR4 recruitment to lipid rafts[247]. Immunoregulatory effects of CO on adaptive immune cells have also been described. CO is able to inhibit T_H cell activation[248] and induce apoptosis in Jurkat T cells[249].

Labile Fe, another end product of heme catabolism by HO-1, affords cytoprotection by inducing the expression of FtH[250]. The Fe storage ability of FtH underlies its ability to prevent TNF-mediated programmed cell death[251].

4. Aims of the Thesis

The aim of the research work presented in this Ph.D Thesis was to understand whether and how the Nrf2 pathway and its downstream gene, *Hmox1*, regulate autoimmune neuroinflammation. For this, we studied the role of Nrf2 expression in the pathogenesis of autoimmune neuroinflammation (*Chapter 2*). Further, we investigated also the role of the expression of the Nrf2-dependent gene, *Hmox1*, in the pathogenesis of autoimmune neuroinflammation (*Chapter 3 and Appendix 1*). We also addressed the significance of induction of HO-1 expression and/or exogenous CO administration from a therapeutic point of view (*Chapter 3*).

Chapter 2

The Transcription Factor Nrf2 Inhibits the Pathogenesis of Autoimmune Neuroinflammation via Regulation of Self-reactive T Helper Type 1 Cell Priming

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1. Abstract

The transcription factor NF-E2-related factor 2 (Nrf2) regulates cellular responses to oxidative stress. Here we investigated the mechanism underlying the protective effect of Nrf2 against autoimmune neuroinflammation. When immunized with myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}), C57BL/6 *Nrf2*-deficient (*Nrf2*$^{-/-}$) mice develop a more severe form of experimental autoimmune encephalomyelitis (EAE), as compared to wild type (*Nrf2*$^{+/+}$) counterparts. This effect is also present when active immunization is bypassed in MOG_{35-55}-specific T cell receptor transgenic (2D2) *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{+/+}$ mice. Dendritic cells (DC) from *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{+/+}$ mice express higher levels of IL-12, increasing the priming of myelin-reactive T helper type 1 (T_H1) cells, that produce IFN-γ. The encephalitogenic effect associated with deletion of the *Nrf2* allele is ablated once IL-12 activity is suppressed using an anti-IL-12/23p40 monoclonal antibody or when IFN-γ activity is suppressed by deletion of the IFN-γ receptor (*Ifngr1*$^{-/-}$). Inhibition of IL-12 production in DC acts via the expression of heme oxygenase-1 (HO-1/*Hmox1*). However, HO-1 expression in DC fails to control myelin-reactive T_H1 cell priming or the pathogenesis of EAE, as demonstrated in mice carrying a deletion of the *Hmox1* allele specifically in DC (*CD11c-Cre/Hmox1*$^{\Delta/\Delta}$) *vs.* control *Hmox1*$^{lox/lox}$ mice. In conclusion, Nrf2 counters the pathogenesis of autoimmune neuroinflammation via a mechanism that involves the inhibition of IL-12 production by DC and thus preventing myelin-reactive T_H1 cell priming. While the immunoregulatory effect of Nrf2 is mediated partially via the expression of HO-1, its overall protective effect against autoimmune neuroinflammation is not fully explained by the expression of HO-1 in DC.

2. Introduction

Multiple sclerosis (MS) is a chronic immune-mediated inflammatory disease of the central nervous system (CNS), affecting about 1 in 1000 individuals in Europe and the US[1]. The pathogenesis of MS is triggered by the activation of myelin-reactive

$CD4^+$ T helper (T_H) cells that establish a chronic inflammatory response in the CNS[2]. Generation of inflammatory and cytotoxic mediators, by macrophages/microglia, in the CNS, causes oligodendrocyte damage, leading to demyelination and eventually to neuronal damage, responsible for the clinical outcome of MS[3].

Much of our current understanding of the mechanisms underlying the pathogenesis of MS was provided by analysis of its animal model, experimental autoimmune encephalomyelitis (EAE)[4]. In a similar manner to MS, the pathogenesis of EAE is driven by the activation of myelin-specific T_H cells that establish focal inflammatory lesions in the CNS, responsible for the clinical signs of the disease[2]. Differentiation of naïve myelin-reactive T_H cells towards an encephalitogenic T_H type 1 (T_H1) phenotype plays a central role in the pathogenesis of EAE as well as MS[1,2].

Presumably, myelin-reactive T_H1 cell priming is triggered by dendritic cells (DC) in the CNS-draining LN[2,5]. Immunogenic DC can present myelin-derived peptides in the context of major histocompatibility complex (MHC) class II[6], while expressing costimulatory molecules and producing cytokines that favor the differentiation of encephalitogenic T_H1 cells[7], characterized by the secretion of interferon (IFN)-γ[8]. The capacity of DC to drive myelin-reactive T_H cell differentiation towards a T_H1 phenotype relies on the production of interleukin (IL)-12p70[9,10].

The encephalitogenic effect of IFN-γ produced by myelin-reactive T_H1 cells is supported by the observation that: i) EAE is associated with high levels of IFN-γ production by CNS-infiltrating T_H1 cells, which is also the case for active MS lesions[11,12], ii) IFN-γ blocking antibodies suppress MS progression[13] and iii) IFN-γ administration exacerbates MS progression[14]. The observation that despite the fact that *Ifng*[15], *Ifngr*[16] and *Il12p35*[17]-deficient mice fail to mount a T_H1 response, these mice remain susceptible to EAE, led to the identification of T_H17 cells, as another major player in the pathogenesis of autoimmune neuroinflammation[18,19].

The mechanisms underlying the encephalitogenicity of myelin-reactive T_H1 and T_H17 cells are probably distinct[20,21]. T_H1 elicit a classical form of EAE with

spinal cord lesions predominating over brain and associated with monocytic infiltrates, while T_H17 induce an atypical form of EAE with brainstem and cerebellum lesions associated with granulocytic infiltrates[20]. When analyzed in conjunction, these forms of EAE recapitulate, to a large extent, the clinical symptoms of MS[4].

The pathologic outcome of autoimmune neuroinflammation reflects the extent of tissue injury imposed to oligodendrocytes and neurons by CNS-infiltrating encephalitogenic T_H1 or T_H17 cells[22]. Therefore, mechanisms inhibiting myelin-reactive T_H1 or T_H17 cell priming should counter the pathogenesis of autoimmune neuroinflammation. Moreover, mechanisms conferring tissue-damage control in the CNS should also contribute to limit disease progression[23]. Presumably, these regulatory mechanisms are mediated by the expression of stress-responsive genes that modulate the pathogenic effect of myelin-reactive T_H cells and/or that confer tissue-damage control in the CNS. We reasoned that some of these stress-responsive genes may fall under the control of the nuclear factor erythroid (NF-E) 2-related factor 2 (Nrf2), a master regulator of adaptive cellular responses to oxidative stress[24,25].

Nrf2 belongs to the Cap'n'collar basic leucine zipper family of transcription factors[24,26] and responds to oxidative stress, sensed by its constitutive repressor Kelch-like ECH-associated protein 1 (Keap1)[26]. Accumulation of free radicals disrupts Keap1 interaction with Nrf2[27], overcoming the constitutive ubiquitination and degradation of Nrf2 by the 26s proteasome[28]. This allows for Nrf2 nuclear translocation and binding to antioxidant response elements (ARE) in the promoter of genes[24] encoding antioxidant and detoxifying enzymes, that provide cellular adaptation to oxidative stress[24]. These oxidative stress-regulated genes include *Hmox1*, encoding the heme-catabolizing enzyme heme oxygenase-1 (HO-1)[29] that confers protection against immune-mediated inflammatory diseases[30], including autoimmune neuroinflammation[31,32].

Deletion of the *Nrf2* allele in mice[25] promotes the development of autoimmune diseases[33-35], including autoimmune neuroinflammation[36,37]. The clinical relevance of these findings is supported by the observation that

polymorphisms reducing NRF2 expression[38] are associated with increased severity of immune-mediated inflammatory diseases[39-41].

Here we report that Nrf2 restrains IL-12 production by DC, limiting myelin-reactive T_H1 cell priming and preventing T_H1 cell-driven IFN-γ from participating in the pathogenesis of autoimmune neuroinflammation. While inhibition of IL-12 by Nrf2 acts via a mechanism involving the expression of HO-1 in DC, inhibition of myelin-reactive T_H1 cell priming or EAE severity by Nrf2 act irrespectively of HO-1 expression in DC.

3. Results

3.1 Nrf2 inhibits the pathogenesis of EAE

We asked whether the onset and/or progression of EAE in MOG_{35-55}-immunized C57BL/6 mice is associated with the expression of Nrf2-regulated genes, as assessed in the CNS at the onset and peak of disease by qRT-PCR. Expression of *Hmox1*, *Ferritin h chain* (*Fth*), *Peroxiredoxin 1* (*Prdx1*), *Superoxide dismutase 2* (*Sod2*), *Glutathione S-transferase*, *theta 1* (*Gstt1*), *Glutathione peroxidase 1* (*Gpx*), *Glutathione reductase* (*Gsr*), *Metallothionein* (*Mt1*), *Metallothionein 2* (*Mt2*), *Thioredoxin 1* (*Trn1*), *Nrf2* and *Cd36* mRNA was induced at the onset and peak of EAE, as compared to naïve mice (*Fig.1a*). Expression of *NADPH dehydrogenase quinone 1* (*Nqo1*) mRNA was induced only at the peak of EAE, while mRNA expression of *Catalase* (*Cat*), *Glutamate-cysteine ligase modifier* (*Gclm*) and *catalytic* (*Gclc*) subunits remained unchanged, as compared to naïve mice (*Fig.1a*). The level of expression of these genes varied in naïve mice (*Suppl. Table 1*), which impacts on their fold-induction, as assessed during EAE. Expression of a subset of these Nrf2 regulated genes, i.e. *Hmox1*[31,32], *Mt1* and *Mt2*[42] has been shown to prevent the pathologic outcome of EAE, supporting the notion that Nrf2 exerts a protective effect against autoimmune neuroinflammation[36,37].

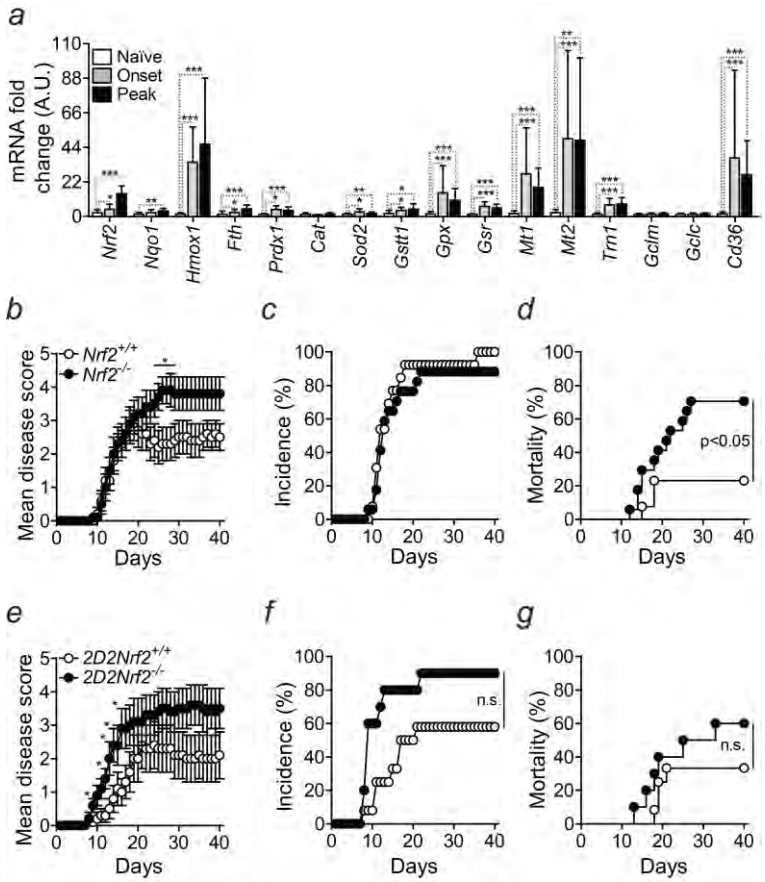

Figure 1. Nrf2 inhibits the pathogenesis of EAE. (***a***) mRNA transcripts were quantified by qRT-PCR in the spinal cord before induction (naïve; white; n=16), at the onset (grey; n=11) or at the peak (black; n=12) of EAE in C57BL/6 mice immunized with MOG_{35-55} in CFA and receiving PTx. Data is shown as mean fold change of mRNA expression relative to naïve mice ± STD. Data was pooled from at least three independent experiments (n=3-6 mice/experiment). (***b***) Mean EAE score ± SEM in wild type (*Nrf2*$^{+/+}$; white; n=13) and *Nrf2*-deficient (*Nrf2*$^{-/-}$; black; n=17) C57BL/6 mice immunized with MOG_{35-55} plus CFA and receiving PTx. Data was pooled from three independent experiments with similar results. (***c***) Disease incidence in the same mice as (b). (***d***) Mortality in the same mice as (b). (**e**) Mean EAE score ± SEM in 2D2*Nrf2*$^{+/+}$ (white, n=12) and 2D2*Nrf2*$^{-/-}$ (black; n=10) C57BL/6 mice receiving PTx. Data was pooled from two independent experiments with similar results. (**f**) Disease incidence in same mice as (e). (***g***) Mortality in same mice as (e). (*) p<0.05; (**) p<0.01; (***) p<0.001.

To assess whether Nrf2 regulates the pathogenesis of autoimmune neuroinflammation, EAE was compared in wild type (*Nrf2*$^{+/+}$) *vs. Nrf2*-deficient (*Nrf2*$^{-/-}$) C57BL/6 mice, immunized with MOG_{35-55} in complete Freund's adjuvant (CFA), and receiving *Pertussis* toxin (PTx). EAE severity was higher in *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{+/+}$ mice (*Fig.1*), illustrated by mean disease scores and incidence of mortality, without changes in disease incidence (*Fig.1b,c,d*). Immunization of *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$

mice with a non-self-protein, i.e. ovalbumin (OVA) in CFA followed by PTx administration, did not lead to mortality (*Suppl. Table 2*). This suggests that Nrf2 confers protection against activation of self-reactive T cells, such as myelin-reactive T_H cells.

Similar results were obtained in 2D2 TCR transgenic mice, in which TCR recognition is restricted to the MOG_{35-55} peptide, when presented in the context of MHC class II[43]. Consistent with previous reports[43], 2D2 mice developed EAE in response to PTx administration, without active immunization (*Fig.1e,f,g*). Disease severity was higher in 2D2 mice lacking Nrf2 (2D2*Nrf2*$^{-/-}$) *vs.* control (2D2*Nrf2*$^{+/+}$) mice, as revealed by mean disease scores (*Fig.1e*). Disease onset occurred earlier in 2D2*Nrf2*$^{-/-}$ *vs.* 2D2*Nrf2*$^{+/+}$ mice, while disease incidence (*Fig.1f*) and mortality (*Fig.1g*) were similar.

The number of CNS-infiltrating leukocytes was similar in polyclonal TCR (*Suppl. Fig.1a-h*) or anti-MOG_{35-55} TCR transgenic (*Suppl. Fig.2a-h*) *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ mice as assessed during EAE. There was an increase in the level of expression of MHC class II by the microglia ($CD45^{low}CD11b^{+}$) (*Suppl. Fig.1d*) as well as a decrease in the number of CNS-infiltrating T_H17 cells (*Suppl. Fig.1f*) in the CNS of *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ mice. This accounts for a slightly higher T_H1 (IFN-γ^{+}) to T_H17 (IL-17A^{+}) cell ratio in *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ (*Suppl. Fig.1g*) as well as in 2D2*Nrf2*$^{-/-}$ *vs.* 2D2*Nrf2*$^{+/+}$ (*Suppl. Fig.2g*) mice, which was not statistically significant. Expression of mRNA encoding a number of immune-related proteins was similar in the CNS of *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ mice, as assessed by qRT-PCR at the onset of EAE (*Suppl. Fig.3*). There were no differences in the number of splenic macrophages, neutrophils, DC, T_H1, T_H17 and T_{REG} cells from *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ mice (*Suppl. Fig.4*), as assessed at the onset of EAE.

3.2. The protective effect of Nrf2 is exerted in the immune system

To determine further the mechanism via which Nrf2 limits EAE severity, leukocytes from immunized 2D2*Nrf2*$^{-/-}$ or 2D2*Nrf2*$^{+/+}$ mice were adoptively transferred into sub-lethally irradiated *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ recipients. Leukocytes from 2D2*Nrf2*$^{-/-}$ *vs.* 2D2*Nrf2*$^{+/+}$ mice elicited higher EAE severity when adoptively transferred to *Nrf2*$^{+/+}$

(*Fig.2a-c*) or *Nrf2*$^{-/-}$ (*Suppl. Fig.5a-c*) recipients, as illustrated by higher mean disease scores (*Fig.2a* and *Suppl. Fig.5a*). Leukocytes from immunized 2D2*Nrf2*$^{-/-}$ mice elicited an earlier onset of disease with similar disease incidence, as compared to 2D2*Nrf2*$^{+/+}$ leukocytes (*Fig.2b* and *Suppl. Fig.5b*). Mortality was higher in mice receiving leukocytes from immunized 2D2*Nrf2*$^{-/-}$ *vs.* 2D2*Nrf2*$^{+/+}$ mice, both for *Nrf2*$^{+/+}$ (*Fig.2c*) and *Nrf2*$^{-/-}$ (*Suppl. Fig.5c*) recipients. EAE severity was not increased in *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ recipient mice, adoptively transferred with leukocytes from 2D2*Nrf2*$^{+/+}$ or 2D2*Nrf2*$^{-/-}$ mice, as illustrated by the mean disease scores (*Fig.2d* and *Suppl. Fig.5d*). Incidence of disease (*Fig.2e* and *Suppl. Fig.5e*) and mortality (*Fig.2f* and *Suppl. Fig.5f*) were similar when leukocytes from immunized 2D2*Nrf2*$^{+/+}$ or 2D2*Nrf2*$^{-/-}$ mice were adoptively transferred into *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ mice. These observations reveal that the protective effect of Nrf2 is exerted via a mechanism controlling leukocyte encephalitogenicity.

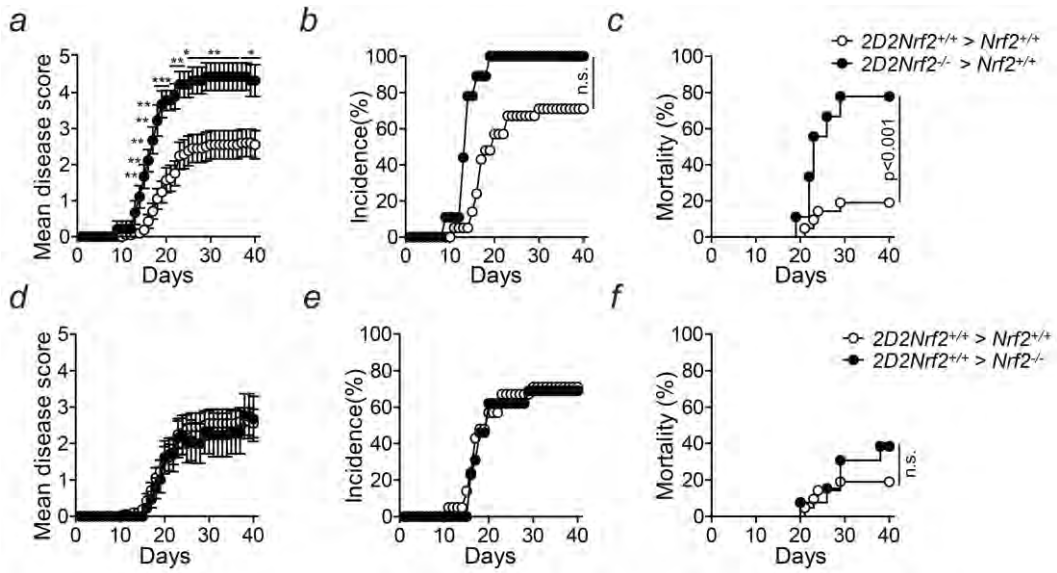

Figure 2. Nrf2 acts in leukocytes to inhibit the pathogenesis of EAE. (***a***) Mean EAE score ± SEM in *Nrf2*$^{+/+}$ mice adoptively transferred with leukocytes from MOG_{35-55}-immunized 2D2*Nrf2*$^{+/+}$ (white; n=21) or 2D2*Nrf2*$^{-/-}$ (black; n=9) mice. (***b***) Disease incidence in the same mice as (a). (***c***) Mortality in the same mice as (a). (***d***) Mean EAE score ± SEM in *Nrf2*$^{+/+}$ (white, n=21) and *Nrf2*$^{-/-}$ (black; n=13) mice adoptively transferred with leukocytes from MOG_{35-55}-immunized 2D2*Nrf2*$^{+/+}$ mice. (***e***) Disease incidence in the same mice as (d). (***f***) Mortality in the same mice as (d). Data was pooled from five independent experiments with similar results. (*) p<0.05; (**) p<0.01; (***) p<0.001.

3.3. Nrf2 regulates cytokine production by activated leukocytes

To determine whether Nrf2 modulates the activation, proliferation and/or differentiation of myelin-reactive T_H cells, proliferation and cytokine production were analyzed in draining LN leukocytes isolated from MOG_{35-55}-immunized *2D2Nrf2*$^{+/+}$ *vs. 2D2Nrf2*$^{-/-}$ mice after MOG_{35-55} re-stimulation *in vitro*. Proliferation of *2D2Nrf2*$^{+/+}$ *vs. 2D2Nrf2*$^{-/-}$ leukocytes was similar, as assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) staining (*Fig.3a,b*). Production of IFN-γ, IL-17A and IL-13 was higher in *2D2Nrf2*$^{-/-}$ vs. *2D2Nrf2*$^{+/+}$ leukocytes, as assessed by ELISA (*Fig.3c*). There was no difference in the production of other cytokines involved in the pathogenesis of EAE, such as TNF, IL-10 or granulocyte macrophage colony-stimulating factor (GM-CSF) (*Fig.3c*). Similar results were obtained in leukocytes isolated from the LN of MOG_{35-55}-immunized *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice, as assessed by [^{3}H]-thymidine incorporation for T_H cell proliferation (*Fig.3d*). Higher production of IFN-γ was confirmed in *Nrf2*$^{-/-}$ vs. *Nrf2*$^{+/+}$ mice leukocytes, as assessed by ELISA (*Fig.3e*). Production of IL-17A and IL-13 were not modulated in *Nrf2*$^{-/-}$ *vs*. *Nrf2*$^{+/+}$ leukocytes (*Suppl. Fig.6*). These observations suggest that Nrf2 inhibits the differentiation of myelin-reactive T_H cells towards an IFN-γ-producing T_H1 phenotype, irrespectively of T_H cell proliferation.

3.4. Nrf2 inhibits IL-12 production by DC

We then asked whether Nrf2 regulates gene expression in DC. Steady state expression of mRNA encoding several Nrf2-regulated genes, such as *Gsr* (*Fig.4e*), *Fth* (*Fig.4f*), *Nqo1* (*Fig.4g*), *Hmox1* (*Fig.4i*) and *Nrf2* itself (*Fig.4h*) was reduced in splenic DC ($CD11c^+MHCII^+$) from *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ mice, as assessed by qRT-PCR. Steady state expression of mRNA encoding *Gpx* (*Fig.4j*) was increased in *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ DC. Bacterial lipopolysaccharide (LPS) induced the expression of *Gsr* (*Fig.4e*) and *Fth* (*Fig.4f*), while reducing the expression of *Hmox1* (*Fig.4i*)[44] and *Gpx* (*Fig.4j*) in *Nrf2*$^{+/+}$ DC, as compared to non-stimulated *Nrf2*$^{+/+}$ DC. Expression of *Nqo1* (*Fig.4g*) and *Nrf2* (*Fig.4h*) remained unchanged in *Nrf2*$^{+/+}$ DC exposed to LPS. Induction of *Gsr* (*Fig.4e*) and *Fth* (*Fig.4f*) in response to LPS was suppressed

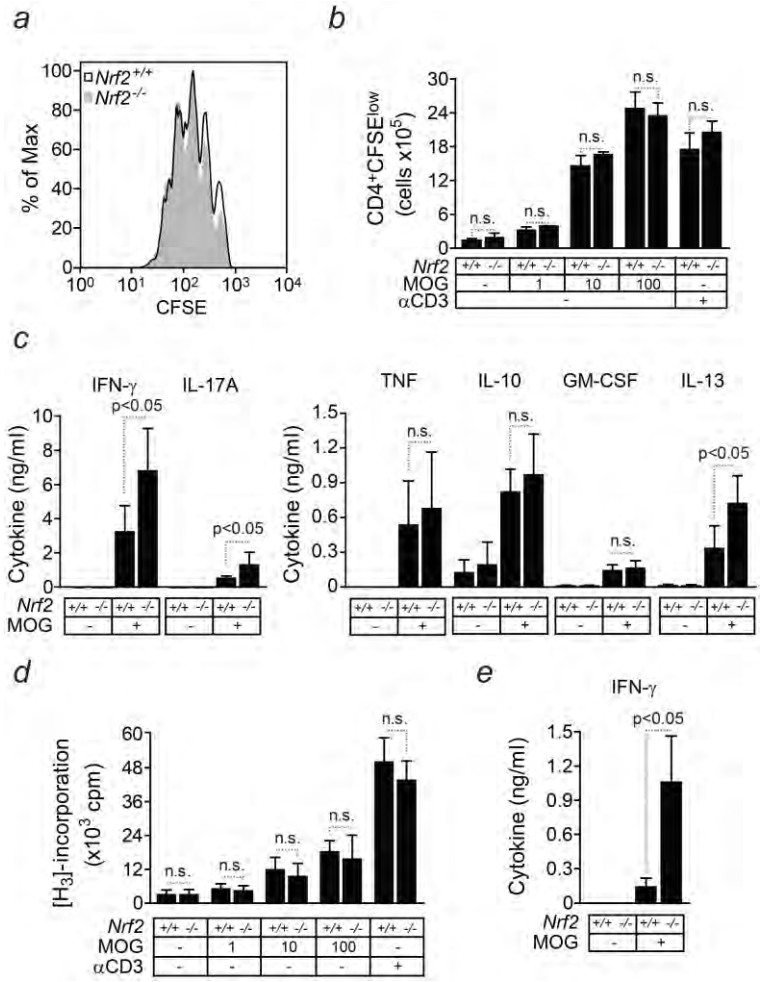

Figure 3. Nrf2 inhibits the production of IFN-γ by MOG_{35-55}-reactive leukocytes. (***a***) Proliferation (CFSE dilution profile) of LN (inguinal and popliteal) leukocytes isolated from MOG_{35-55}-immunized 2D2*Nrf2*$^{+/+}$ (open black line) and 2D2*Nrf2*$^{-/-}$ (filled grey) and re-challenged *in vitro* with MOG_{35-55} (100 μg/ml). Histogram represents cells from representative mice out of 3-4, in one out of two independent experiments. (***b***) Number of proliferating $CD4^{+}CFSE^{low}$ cells from immunized 2D2*Nrf2*$^{+/+}$ (n=4) or 2D2*Nrf2*$^{-/-}$ (n=3) mice, re-challenged *in vitro* with MOG_{35-55} (1, 10, 100 μg/ml) or an anti-CD3 mAb (+; 0.5 μg/ml). Data is shown as mean ± STD from individual mice (n=3-4) from two independent experiments. (***c***) Cytokine concentration in supernatants from LN leukocytes isolated from 2D2*Nrf2*$^{+/+}$ (n=11) or 2D2*Nrf2*$^{-/-}$ (n=11) mice immunized with MOG_{35-55} in CFA and cultured *in vitro* with MOG_{35-55} (+; 100 μg/ml). Data is shown as mean ± SEM from three independent experiments, performed in triplicate (n=3-5 mice/group). (***d***) Proliferation ($[^3H]$-thymidine incorporation) of LN leukocytes isolated from MOG_{35-55}-immunized *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ mice and cultured *in vitro* with MOG_{35-55} (1, 10, 100 μg/ml) or anti-CD3 mAb (+; 0.5 μg/ml). Data is shown as mean counts per minute (cpm) ± STD from individual mice (n=6) in two independent experiments with similar trend. (***e***) IFN-γ concentration in supernatants from leukocytes isolated from the 46 of MOG_{35-55}-immunized *Nrf2*$^{+/+}$ (n=8) or *Nrf2*$^{-/-}$ (n=8) mice cultured *in vitro* with MOG_{35-55} (+; 100 μg/ml). Data is shown as mean ± STD from individual mice in two independent experiments (n=3-5 mice/group), with similar trend.

in *Nrf2*$^{-/-}$ DC. Expression of *Nqo1* (*Fig.4g*) and *Hmox1* (*Fig.4i*) were suppressed in *Nrf2*$^{-/-}$ *vs.* Nrf2$^{+/+}$ DC. This suggests that Nrf2 regulates gene expression in naïve as well as LPS-activated splenic DC.

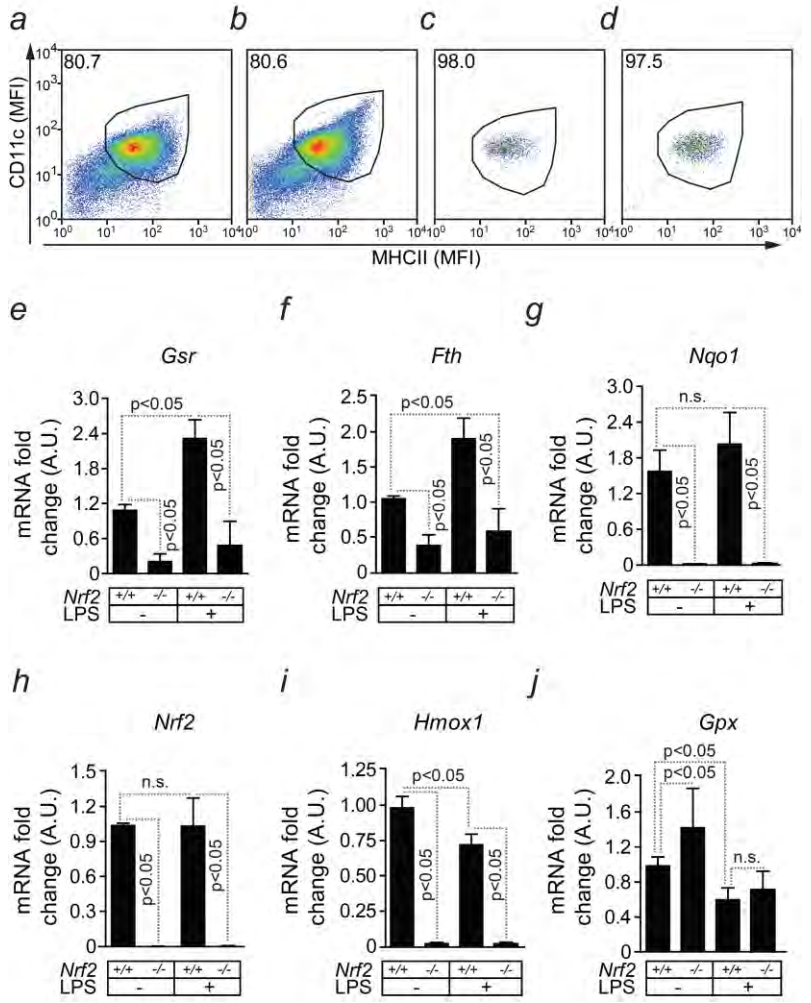

Figure 4. Nrf2 regulates gene expression in DC. (***a***) Representative dot plots of splenic DC (CD11c^{+}MHCII^{+}) from (***a***) *Nrf2*$^{+/+}$ and (***b***) *Nrf2*$^{-/-}$ mice, purified using anti-CD11c magnetic beads. Representative dot plots of splenic (***c***) *Nrf2*$^{+/+}$ and (***d***) *Nrf2*$^{-/-}$ DC sorted as CD11c^{+}MHCII^{+}TCRβ^{-}IgM^{-}CD19^{-} cells. Expression of (***e***) *Gsr*, (***f***) *Fth*, (***g***) *Nqo1*, (***h***) *Nrf2,* (***i***) *Hmox1* and (***j***) *Gpx* mRNA analyzed by qRT-PCR in splenic DC (CD11c^{+}MHCII^{+}TCRβ^{-}IgM^{-}CD19^{-}) from *Nrf2*$^{+/+}$ C57BL/6 mice as in (c) or *Nrf2*$^{-/-}$ as in (d), cultured in presence (+) or absence (-) of LPS (100 ng/ml; 4h). Data is shown as mean fold change of mRNA expression relative to wild type (*Nrf2*$^{+/+}$) naïve cells ± STD from four independent experiments with similar trend (n=3-4 pooled mice/genotype/experiment).

We then asked whether Nrf2 modulates the immunogenic effect of DC, in particular related to their capacity to produce IL-12, required to prime myelin-reactive T_H1 cells. Expression of *Il12/23p40* mRNA was increased in naïve splenic *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ DC as well as in response to LPS *in vitro*, as assessed by qRT-PCR (*Fig.5a*). This effect was confirmed for IL-12/23p40 protein expression by ELISA in splenic *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ DC exposed *in vitro* to different toll like receptor agonists, including LPS (TLR4), Poly(I:C) (TLR3) or CpG (TLR9)(*Fig.5b*). Production of IL-12p40 protein was also higher in *Nrf2*$^{-/-}$ *vs*. *Nrf2*$^{+/+}$ Flt3 ligand (Flt3L)-derived bone marrow DC, stimulated *in vitro* with LPS or Poly(I:C) but not with CpG (*Suppl. Fig.7*). Expression of *Il12p35* mRNA in response to LPS was also higher in splenic *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ DC, as assessed by qRT-PCR (*Fig.5c*). This was also the case for IL-12p70, resulting from IL-12/23p40 and IL-12p35 heterodimerization, as assessed by ELISA (*Fig.5d*). Expression of *Il23p19* mRNA remained unchanged in *Nrf2*$^{-/-}$ *vs*. *Nrf2*$^{+/+}$ DC, both at steady state and after LPS stimulation, as assessed by qRT-PCR (*Suppl. Fig.8*). IL-23 protein was undetectable in cell culture supernatants from splenic DC, as assessed by ELISA. These observations demonstrate that Nrf2 inhibits the production of IL-12 in DC.

Induction of IL-6 expression in response to LPS was abolished in splenic DC from *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ mice (*Fig.5e*), which is in keeping with the notion that Nrf2 controls *Il6* transcription in other cell types[45]. Induction of TNF expression by LPS was also reduced in splenic *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ DC (*Fig.5f*).

Expression of surface molecules regulating DC immunogenicity, including MHC class II, CD40, CD80, CD86 and CD70 was similar in splenic *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ DC, both at steady state or after LPS stimulation (*Suppl. Fig.9a*). The same is true for splenic DC analyzed at the onset of EAE in *Nrf2*$^{+/+}$ *vs*. *Nrf2*$^{-/-}$ mice (*Suppl. Fig.9b*). This demonstrates that Nrf2 inhibits IL-12 production by DC, while promoting IL-6 and TNF production, suggesting that Nrf2 might inhibit T_H1 priming, a process which requires the production of IL-12 by DC[9,10].

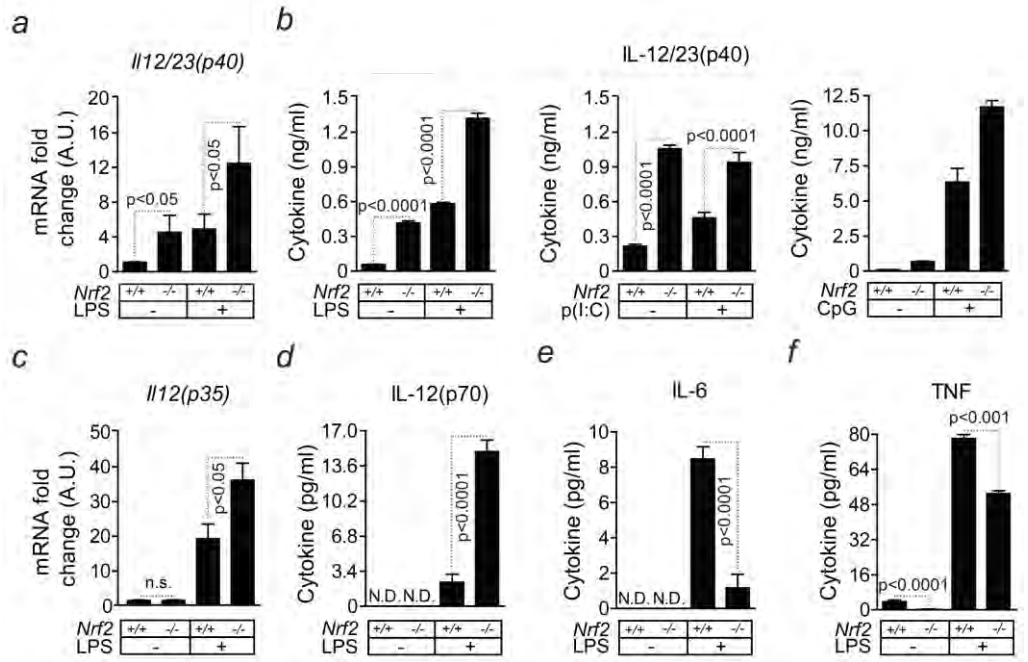

Figure 5. Nrf2 inhibits IL-12 and promotes IL-6 and TNF expression in DC. (***a***) Expression of *Il12/23p40* analyzed by qRT-PCR in sorted ($CD11c^+MHCII^+TCR\beta^-IgM^-CD19^-$) splenic DC from *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ C57BL/6 mice cultured in presence (+) or absence (-) of LPS (100 ng/ml; 4h). Data is shown as mean ± STD from four independent experiments, with similar trend (n=3-4 pooled mice/genotype/experiment). (***b***) Concentration of IL-12/23p40, in cell culture supernatants of sorted splenic DC stimulated with LPS (+; 100 ng/ml), Poly(I:C) (+; 50 μg/ml) or CpG (+; 0.1μM) for 24h. Data is shown as mean ± STD from 5 (LPS and PolyI:C) or 3 wells (CpG) in one experiment representative of at least two independent experiments with similar trend (n=3-4 pooled mice/genotype/experiment). (***c***) Expression of *Il12p35* analyzed by qRT-PCR in sorted splenic DC as in (a). Data is shown as mean ± STD from three independent experiments, with similar trend (n=3-4 pooled mice/genotype/experiment). (***d***) IL-12p70, (***e***) IL-6 and (***f***) TNF in cell culture supernatants of sorted splenic DC stimulated with LPS (+; 100 ng/ml; 24h) as in (*b*). Data is shown as mean ± STD from one representative experiment performed in sixtuplicate out of two (TNF and IL-6) or three (IL-12p70) independent experiments with similar trend (n=3-4 pooled mice/genotype/experiment).

3.5. Nrf2 inhibits T_H1 priming by DC

We hypothesized that expression of Nrf2 in DC inhibits the differentiation of naïve myelin-reactive T_H cells towards an IFN-γ-secreting T_H1 phenotype (*Fig.3c,e*). Naïve 2D2*Nrf2*$^{+/+}$ or 2D2*Nrf2*$^{-/-}$ T_H cells were primed *in vitro* with MOG_{35-55}-pulsed splenic *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ DC, activated or not by LPS. Cytokines were measured by ELISA in cell culture supernatants after re-challenge with an anti-CD3 monoclonal antibody (mAb). 2D2 T_H cells produced higher levels of IFN-γ when primed with *Nrf2*$^{-/-}$ *vs.* *Nrf2*$^{+/+}$ DC (*Fig.6a*). 2D2 T_H cells from *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ mice produced similar levels of IFN-γ in response to priming by *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ DC (*Fig.6a* and *Suppl. Fig.10a*).

Nrf2 expression in DC or T_H cells had no effect on the production of other cytokines, such as IL-17A (*Fig.6b*), TNF (*Suppl. Fig.10d* and *11a*), IL-2 (*Suppl. Fig.10e* and *11b*), IL-10 (*Suppl. Fig.10f* and *11c*), GM-CSF (*Suppl. Fig.10c* and *12a*) or IL-13 (*Suppl. Fig.10g* and *12b*). These observations suggest that expression of Nrf2 in DC inhibits the priming of naïve MOG_{35-55}-reactive T_H cells towards IFN-γ-producing T_H1 cells. Of notice, 2D2 T_H cells from *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ mice produced higher levels of IL-4 when primed by *Nrf2*$^{+/+}$ DC (*Suppl. Fig.10h*).

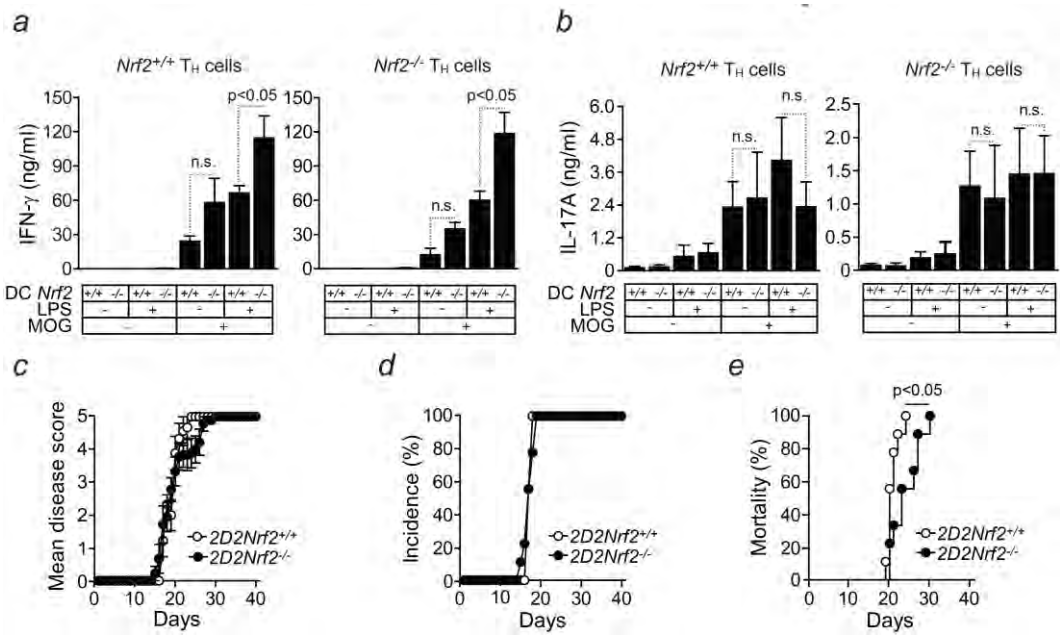

Figure 6. The protective effect of Nrf2 is mediated via inhibition of IFN-γ production by myelin-reactive T_H1 cells. Concentration of (***a***) IFN-γ and (***b***) IL-17A in cell culture supernatants of T_H cells from naïve 2D2*Nrf2*$^{+/+}$ or 2D2*Nrf2*$^{-/-}$ mice cultured with MOG_{35-55}-pulsed splenic DC from *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ mice (96h). When indicated (+), DC were stimulated with LPS (100 ng/ml). Cytokine production was assessed 48h after re-stimulation with an anti-CD3 (2.5 μg/ml) mAb. Data is shown as mean ± SEM pooled from at least three independent experiments with similar trend, each performed at least in quintuplicate. (***c***) Mean EAE score ± SEM in *Ifngr1*$^{-/-}$ mice adoptively transferred with leukocytes isolated from MOG_{35-55}-immunized 2D2*Nrf2*$^{+/+}$ (white; n=9) or 2D2*Nrf2*$^{-/-}$ (black; n=9). Results were pooled from two independent experiments with similar trend. (***d***) Disease incidence in the same mice as (c). (***e***) Mortality in the same mice as (c).

3.6. Nrf2 inhibits the encephalitogenic effect of IFN-γ produced by myelin-reactive T_H1 cells

We reasoned that if inhibition of T_H1 cell priming is functionally involved in the protective effect of Nrf2 against EAE, this effect should be abrogated in mice in which the biologic activity of IFN-γ is suppressed. To test this hypothesis,

leukocytes from MOG_{35-55}-immunized 2D2*Nrf2*$^{+/+}$ and 2D2*Nrf2*$^{-/-}$ mice were adoptively transferred into recipients that do not express the IFN-γ receptor (*Ifngr1*$^{-/-}$). Consistent with previous reports[46], adoptive transfer of leukocytes into naïve *Ifngr1*$^{-/-}$ mice led to the development of an atypical axial-rotatory form of EAE. Severity of EAE was similar in *Ifngr1*$^{-/-}$ recipient mice receiving 2D2*Nrf2*$^{+/+}$ *vs.* 2D2*Nrf2*$^{-/-}$ leukocytes, as revealed by mean disease scores (*Fig.6c*) and incidence of disease (*Fig.6d*). Onset of mortality was slightly delayed in *Ifngr1*$^{-/-}$, receiving leukocytes from 2D2*Nrf2*$^{-/-}$ *vs.* 2D2*Nrf2*$^{+/+}$ mice (*Fig.6e*). These observations suggest that the protective effect of Nrf2 against EAE is mediated via a mechanism involving the inhibition of IFN-γ production by myelin-reactive T_H1 cells.

3.7. Nrf2 controls T_H1 encephalitogenicity via inhibition of IL-12 production in DC

Given that Nrf2 inhibits the expression of IL-12 in DC, we asked whether this effect would be sufficient to impair myelin-reactive T_H cell priming towards IFN-γ-producing T_H1 cells. Inhibition of IL-12 activity using a neutralizing anti-IL-12/23p40 mAb restored the levels of IFN-γ produced by T_H cells primed *in vitro* with MOG_{35-55}-pulsed splenic *Nrf2*$^{-/-}$ DC to those of T_H cells primed *in vitro* with MOG_{35-55}-pulsed splenic *Nrf2*$^{+/+}$ DC (*Fig.7a*). This effect was not observed using an isotype-matched control mAb (*Fig.7a*). This suggests that expression of Nrf2 in DC inhibits the differentiation of encephalitogenic T_H1 cells via a mechanism that relies on the inhibition of IL-12 production by DC. Production of IL-17A was not modulated by the anti-IL-12/23p40 neutralizing mAb, as compared to an isotype-matched control mAb (*Fig.7b*).

We then tested whether neutralization of IL-12 *in vivo* reduces EAE severity in 2D2*Nrf2*$^{-/-}$ mice. Administration of an anti-IL-12/23p40 neutralizing mAb to 2D2*Nrf2*$^{-/-}$ mice suppressed the onset of EAE in response to PTx administration, as compared to an isotype-matched control mAb (*Fig.7c-e*). This is revealed by a reduction in mean disease scores (*Fig.7c*), incidence of disease (*Fig.7d*) and mortality (*Fig.7e*). A similar effect was observed in 2D2*Nrf2*$^{+/+}$ mice (*Suppl. Fig.13*).

This reveals that the onset of EAE in 2D2 mice receiving PTx is strictly controlled by IL-12 and/or IL-23, suggesting that inhibition of IL-12 contributes to the protective effect of Nrf2 against autoimmune neuroinflammation.

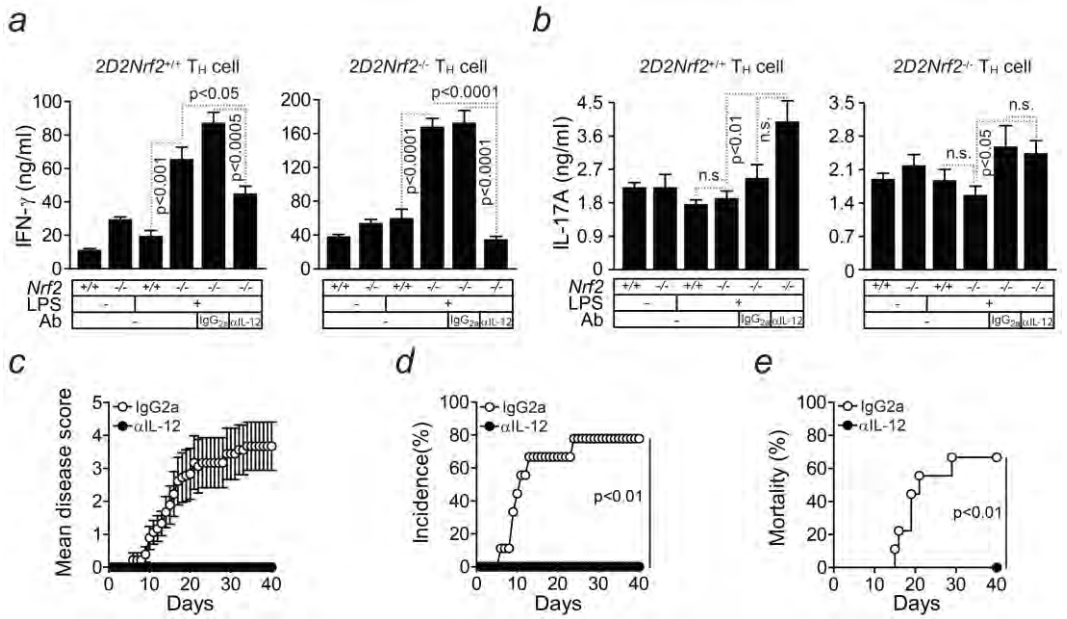

Figure 7. The protective effect of Nrf2 is mediated via inhibition of IL-12/23p40 production. Concentration of (***a***) IFN-γ and (***b***) IL-17A in cell culture supernatants of T_H cells from naïve 2D2*Nrf2*$^{+/+}$ or 2D2*Nrf2*$^{-/-}$ mice co-cultured with MOG_{35-55}-pulsed splenic DC from *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ mice (96h). When indicated (+), DC were stimulated with LPS (100 ng/ml) and an anti-IL-12/23p40 neutralizing antibody (αIL-12; 20 μg/ml) or an isotype-matched control antibody (IgG2a; 20 μg/ml) were added to the cell culture. Cytokine production was assessed 48h after re-stimulation with an anti-CD3 (2.5 μg/ml) mAb. Data is shown as mean ± STD of one experiment representative of two independent experiments with similar trend, performed at least in quintuplicate. (***c***) Mean EAE score ± SEM in 2D2*Nrf2*$^{-/-}$ treated with an anti-IL-12/23p40 neutralizing antibody (αIL-12; black; n=8) *vs.* an isotype-matched control antibody (IgG2a; white; n=9). Data is pooled from three independent experiments with similar trend. (***d***) Disease incidence in the same mice as (c). (***e***) Mortality in the same mice as (c).

3.8. The protective effect of Nrf2 is not mediated via the expression of HO-1 in DC

Nrf2 controls the expression of HO-1 in several cell types[29], including DC (*Fig.4i*). Given that expression of HO-1 inhibits the pathological outcome of EAE[31,32,47], we reasoned that the protective effect of Nrf2 against EAE might act via a mechanism involving the induction of HO-1 expression in DC. Expression of IL-12p70 was higher in both naïve and LPS-stimulated splenic *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ DC, as assessed *in vitro* (*Fig.8a*). This indicates that Nrf2 inhibits the expression of IL-

12p70 in DC most probably via a mechanism involving the expression of one of its target genes, namely *Hmox1*.

In a similar manner to Nrf2 (*Suppl. Fig.9a*), HO-1 failed to regulate the expression of MHC class II (IAd) and costimulatory molecules, regulating DC immunogenicity, including CD40, CD80 and CD86, as assessed *in vitro* in unstimulated or LPS-stimulated GM-CSF bone marrow-derived DC from *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ mice (*Suppl. Fig.14*) or *in vivo* in splenic DC from naïve or LPS-stimulated *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ mice (*Suppl. Fig.15*). This indicates that when expressed under physiologic conditions in DC, HO-1 does not regulate DC maturation, as assessed by the expression of MHC class II and costimulatory molecules.

In a similar manner to Nrf2 (*Fig.3a,b,d*), expression of HO-1 in DC also failed to regulate T cell proliferation, as assessed in leukocytes isolated from OVA-immunized *Hmox1*$^{+/+}$ *vs. Hmox1*$^{-/-}$ mice, re-challenged with OVA *in vitro* (*Fig.8b*). This is in keeping with our previous finding[48], showing that when expressed under physiologic conditions HO-1 does not regulate T_H cell proliferation.

We then asked whether expression of HO-1 regulates naïve T_H cell priming towards an IFN-γ-secreting T_H1 phenotype. Leukocytes from OVA-immunized *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ mice produced similar levels of IFN-γ when re-stimulated with OVA *in vitro* (*Fig.8c*). This indicates that Nrf2 inhibits T_H1 cell differentiation via a mechanism that does not involve HO-1.

Finally, we asked whether expression of HO-1 in DC contributes to the protective effect of Nrf2 against autoimmune neuroinflammation. EAE severity was slightly but not significantly higher in MOG_{35-55}-immunized C57BL/6 *Hmox1*$^{lox/lox}$ mice, carrying a functional *Hmox1* allele flanked by *LoxP* sites, as compared to *CD11c-Cre/Hmox1*$^{\Delta/\Delta}$ mice in which the *Hmox1*$^{lox/lox}$ allele is deleted by the expression of Cre recombinase under the control of the *CD11c* promoter[49]. This is revealed by the mean disease scores (*Fig.8d*), incidence of disease (*Fig.8e*) and mortality (*Fig.8f*). *Hmox1* deletion in splenic DC from *CD11c-Cre/Hmox1*$^{\Delta/\Delta}$ *vs. Hmox1*$^{lox/lox}$ was confirmed by qRT-PCR (*Suppl. Fig.16*). These experiments reveal

that *Hmox1* deletion in DC is not sufficient *per se* to impact on the pathologic outcome of EAE, suggesting that the protective effect of Nrf2 against autoimmune neuroinflammation is not mediated by the induction of HO-1 expression in DC.

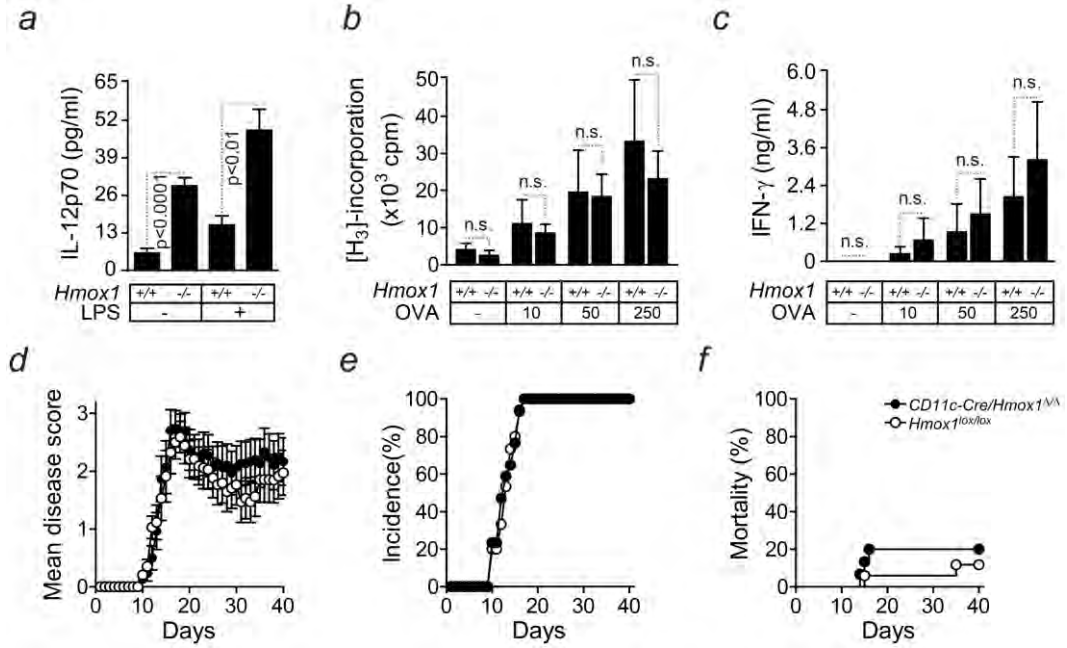

Figure 8. The protective effect of Nrf2 is not mediated by expression of HO-1 in DC. (***a***) Splenic DC from BALB/c *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mice were stimulated *in vitro* with LPS (+; 100 ng/ml). Supernatants were collected 24 hours thereafter and IL12p70 measured by ELISA. Data is pooled from 5 independent experiments performed in sixtuplicate and shown as mean ± SEM. (***b***) Proliferation of LN leukocytes from BALB/c *Hmox1*$^{+/+}$ (n=6) or *Hmox1*$^{-/-}$ (n=6) mice harvested eight days after footpad immunization with OVA, emulsified in CFA (2 mg/ml; 50 μl of emulsion/footpad). Cells were re-stimulated *in vitro* with OVA (10, 50, 250 μg/ml) for 72h and [^{3}H]-thymidine incorporation was measured. Data is shown as mean ± STD (n=6 mice/group) from two independent experiments with similar results. (***c***) IFN-γ production by LN leukocytes isolated from BALB/c *Hmox1*$^{+/+}$ (n=9) or *Hmox1*$^{-/-}$ (n=9) mice 8 days after footpad immunization with OVA emulsified in CFA. Leukocytes were re-stimulated *in vitro* with OVA (10, 50, 250 μg/ml) for 72h and IFN-γ was measured in cell culture supernatants by ELISA. Data is shown as mean ± STD (n=9 mice/group) from three independent experiments with similar results. (***d***) Mean EAE score ± SEM in *Hmox1*$^{lox/lox}$ (white; n=17) *vs. CD11c-Cre/Hmox1*$^{\Delta/\Delta}$ (black; n=15) C57BL/6 mice immunized with MOG_{35-55} plus CFA and receiving PTx. Data was pooled from three independent experiments with similar results (***e***) Disease incidence in the same mice as (e). (***f***) Mortality in the same mice as (e).

4. Discussion

DC play a central role in the initiation of both protective and pathologic T_H cell responses, including those involved in the pathogenesis of autoimmune neuroinflammation[50,51]. Presumably, this pathologic effect is related to the inherent capacity of DC to activate naïve myelin-reactive T_H cells[50] in the periphery as well as to reactivate primed T_H cells thereafter in the CNS[52]. Expression of IL-12 by DC,

is necessary and sufficient to drive the differentiation of naïve myelin-reactive T_H cells towards the encephalitogenic T_H1 phenotype[9,10]. That IL-12 contributes in a critical manner to the pathogenesis of autoimmune neuroinflammation is supported by the observation that polymorphisms in the human *IL12A* (*IL12p35*), *IL12B* (*IL12/23p40*) and *IL12RB1* genes act as genetic risk factors contributing to the pathogenesis of MS[53]. Presumably therefore, regulatory mechanisms inhibiting the production of IL-12 in DC should restrain the priming of naïve myelin-reactive T_H cells towards a T_H1 phenotype and as such, prevent the pathogenesis of autoimmune neuroinflammation. As demonstrated herein, the transcription factor Nrf2 acts in such a manner.

EAE severity is exacerbated in *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ mice, as assessed both when EAE is induced by active immunization in C57BL/6 mice (*Fig.1b-d*) or without active immunization in transgenic 2D2 mice (*Fig.1e-f*). This suggests that the protective effect of Nrf2 against autoimmune neuroinflammation can act under pathophysiologic conditions, i.e. without active immunization, such as those associated with the pathogenesis of MS. In keeping with this notion is the finding that Dimethylfumarate, a synthetic pharmacological molecule that activates Nrf2, affords protection against EAE[54] as well as MS[55]. Given the above, understanding the mechanism of action of Nrf2 should be relevant for the treatment of MS.

Nrf2 is a transcription factor that provides cellular metabolic adaptation to oxidative stress[24]. While protective against autoimmune neuroinflammation[36,37] (*Fig.1b-g*), the mechanism underlying the salutary effect of Nrf2 does not seem to operate in resident cells of the CNS. Instead, Nrf2 exerts an immunoregulatory effect that restrains the capacity of DC to prime naïve myelin-reactive T_H1 cells involved in the pathogenesis of autoimmune neuroinflammation. This notion is supported by the following observations. First, adoptive transfer of leukocytes from MOG_{35-55}-immunized *Nrf2*$^{-/-}$ mice elicits a more severe form of EAE, as compared to adoptive transfer of *Nrf2*$^{+/+}$ leukocytes (*Fig.2a-c*). The reverse is not true, in that adoptive transfer of leukocytes from MOG_{35-55}-immunized mice elicits similar EAE severity in *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ recipient mice (*Fig.2d-e*). Second, expression of Nrf2 in DC inhibits the production of IL-12p70, as demonstrated in *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ DC

(*Fig.5d*). Third, Nrf2 prevents the differentiation of naïve myelin-reactive T_H cells towards an encephalitogenic T_H1 phenotype (*Fig.6a*), an effect determined solely by the expression of this transcription factor in DC, as demonstrated using *Nrf2*$^{+/+}$ *vs.* *Nrf2*$^{-/-}$ DC (*Fig.6a*). Fourth, inhibition of T_H1 cell differentiation using an IL-12/23p40 neutralizing antibody (*Fig.7c-e*) or inhibition of T_H1 cell effector function in *Ifngr1*-deficient mice (*Fig.6c-e*) ablates the pathogenic effect associated with *Nrf2* deletion. These findings provide a mechanism of action for the protective effect of Nrf2 against autoimmune neuroinflammation[36,37], contributing to explain how therapeutic molecules, such as Dimethylfumarate, act via this signal transduction pathway to prevent the pathogenesis and/or progression of MS.

Expression of Nrf2 inhibits IL-12 (*Fig.5a-d*), while promoting IL-6 and TNF (*Fig.5e,f*) expression in DC. This effect should not only inhibit the differentiation of naïve myelin-reactive T_H cells towards a T_H1 phenotype, but also promote T_H17 differentiation. This appears to be the case for T_H1 cells, as demonstrated by the observation that blocking IL-12/23p40 *in vitro* is sufficient to reverse the T_H1 bias associated with deletion of the *Nrf2* allele in DC (*Fig.7a*). However, the observation that Nrf2 does not regulate the number of IFN-γ-producing T_H1, or of T_H17 cells infiltrating the CNS of mice undergoing EAE is difficult to conciliate with this notion (*Suppl. Fig.1* and *2*). It is possible that this immunoregulatory effect is masked in the CNS by the conversion of infiltrating T_H17 cells into IFN-γ-producing T_H cells that become a major source of IFN-γ in the CNS during EAE[56]. Presumably, Nrf2 prevents the priming of IFN-γ-producing T_H1 cells, while not affecting the later conversion of T_H17 cells into IFN-γ-producing T_H cells. It should be noted that the number of T_H17 cells in the CNS of *Nrf2*$^{-/-}$ mice is reduced as compared to *Nrf2*$^{+/+}$ mice undergoing EAE (*Suppl. Fig.1f*), which is consistent with the reduction of IL-6 production by DC.

Presumably, the molecular mechanism via which Nrf2 controls the expression of IL-12 in DC is mediated via the expression of immunoregulatory genes controlled by Nrf2. One of the Nrf2-responsive genes that could contribute to this effect is *Hmox1* (*Fig.4i*), which encodes an enzyme that catabolizes free heme

into labile iron and biliverdin, while producing carbon monoxide (CO)[57]. The observation that *Hmox1*$^{-/-}$ DC produce higher levels of IL-12p70 *in vitro*, as compared to *Hmox1*$^{+/+}$ DC (*Fig.8a*), suggests that Nrf2 inhibits the expression of IL-12 via the induction of HO-1 expression in DC (*Fig.4i*). This notion is in keeping with the observation that CO suppresses the pathogenesis of autoimmune neuroinflammation[31] and inhibits IL-12p70 production by DC[58]. Moreover, the protective effect of Dimethylfumarate against EAE also acts via a mechanism involving the expression of Nrf2[36] and HO-1, which inhibits the production of IL-12/23p40 and IL-12p70 in DC[54].

However, deletion of the *Hmox1* allele fails to modulate the differentiation of naïve T_H cells towards a T_H1 phenotype (*Fig.8c*), suggesting that this effect is mediated via other Nrf2-regulated genes. Moreover, specific deletion of the *Hmox1* allele in DC has only a minor, if any, impact in EAE severity (*Fig.8d-f*). This suggests that expression of Nrf2 in DC inhibits myelin-reactive T_H1 cell priming and confers protection against EAE via a mechanism not mediated exclusively by HO-1. Candidate Nrf2-responsive genes include *Mt1 and Mt2*[42].

Several studies have suggested that expression of HO-1 in DC exerts immunosuppressive effects that limit the pathological outcome of immune-mediated inflammatory diseases[47], including autoimmune neuroinflammation[31,32]. It should be noted however, that most of these studies are based on pharmacologic modulation of HO activity[31,44,58], using pharmacologic molecules that can act irrespectively of HO-1[59], which questions the physiologic relevance of these findings. This is in keeping with the observation that when HO-1 is expressed under physiologic conditions in DC, it fails to regulate DC maturation (*Suppl. Fig.14* and *15*) or T cell proliferation (*Fig.8b*). However, HO-1 can inhibit the expression of IL-12 in DC (*Fig.8a*), suggesting that it can exert immunoregulatory effects via its expression in DC.

In conclusion, we demonstrate that the transcription factor Nrf2 exerts immunoregulatory effects in DC that limit the progression of a prototypical T_H1-driven immunopathology, such as demonstrated for autoimmune neuroinflammation in mice. This protective effect is mediated only partially via the expression of HO-1.

Given that pharmacologic inducers of Nrf2 act therapeutically against MS, these findings should be relevant for the treatment of MS.

5. Methods

Mice. Mice were maintained under specific pathogen-free conditions at the Instituto Gulbenkian de Ciência and all experimental protocols were approved by the "Instituto Gulbenkian de Ciência animal care committee" and by the "Direção Geral de Veterinária (DGV)" of the Portuguese Ministry of Agriculture, Rural Development and Fisheries (License 018831-2010-09-03). C57BL/6 *Nrf2*$^{-/-}$[25] and *Hmox1*^{LoxP}[60] mice were generated by the laboratory of Dr. Masayuki Yamamoto (Tohoku University Graduate School of Medicine) and obtained through the RIKEN BioResource Center (Nrf2 knockout mouse/C57BL6J and B6J.129P2-Hmox1<tm1Mym>). *Nrf2*$^{-/-}$ mice were bred at Instituto Gulbenkian de Ciência from *Nrf2*$^{+/-}$ breeding pairs and genotyped by PCR from genomic DNA using the following primers: *Nrf2-5*' 5'-TGGACGGGACTATTGAAGGCTG-3', Lacz 5'-GCGGATTG ACCGTAATGGGATGG-3', AS 5'-GCCGCCTTTTCAGTAGATGGAGG-3'. C57BL/6$^{CD11c\text{-}Cre}$[49] mice were generated by the laboratory of Dr. Boris Reizis (Columbia University Medical Center) and obtained through the Jackson Laboratory (B6.Cg-Tg(Itgax-cre)1-1Reiz/J). *CD11c-Cre/Hmox1*$^{\Delta/\Delta}$ mice were produced at the Instituto Gulbenkian de Ciência from *CD11c-Cre/Hmox1*$^{lox/lox}$ x *Hmox1*$^{lox/lox}$ mice. *CD11c-Cre/Hmox1*$^{\Delta/\Delta}$ and *Hmox1*$^{lox/lox}$ mice were genotyped by PCR from genomic DNA using the following primers: CD11c-Cre transgene forward 5'-ACTTGGCAGCTGTCTCCAAG-3', CD11c-Cre transgene reverse 5'-GCGAACATCTTCA GGTTCTG-3', CD11c-Cre internal control forward 5'-CAAATGTTGCTTGTCTGGTG-3', CD11c-Cre internal control reverse 5'-GTCAGTCGAGTGCACAGTTT-3', *Hmox1* forward 5'-CTCACTATGCAACTCTGTTGGAGG-3', *Hmox1* wt reverse 5'-GTCTGTAATC CTAGCACTCGAA-3' and *Hmox1* flox reverse 5'-GGAAGGACAGCTTCTTGTAGTCG-3'. Deletion of the *Hmox1*$^{lox/lox}$ allele by the Cre recombinase was assessed in sorted splenic DC by qRT-PCR from *CD11c-Cre/Hmox1*$^{\Delta/\Delta}$ relative to *Hmox1*$^{lox/lox}$ mice using the following set of primers: 5'-AGGAGGTACACATCCAAGCCGAG-3' and 5'-

GATATGGTACAAGGAAGCCATCACCAG-3'. BALB/c *Hmox1*$^{+/-}$ mice[61] were generated by Dr. Shaw-Fang Yet (Brigham and Women's Hospital). BALB/c *Hmox1*$^{+/-}$ and BALB/c.SCID.*Hmox1*$^{+/-}$ were bred at the Instituto Gulbenkian de Ciência from *Hmox1*$^{+/-}$ or SCID.*Hmox1*$^{+/-}$ breeding pairs and genotyped as described elsewhere[62]. *Ifngr1*$^{-/-}$[63] mice were generated by the laboratory of Dr. Michel Aguet (Swiss Institute for Experimental Cancer Research) and MOG_{35-55} TCR transgenic mice (2D2)[43] by Dr. Vijay Kuchroo (Brigham and Women's Hospital), both obtained through the Jackson Laboratory (B6.129S7-*Ifngr1*^{tm1Agt}/J and C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J). 2D2*Nrf2*$^{+/-}$ mice were bred from hemizigous 2D2*Nrf2*$^{+/-}$ x *Nrf2*$^{+/-}$ mice and interbred to produce 2D2*Nrf2*$^{+/+}$ and 2D2*Nrf2*$^{-/-}$ mice. 2D2*Nrf2*$^{+/+}$ and 2D2*Nrf2*$^{-/-}$ mice were genotyped by PCR, using the following primers: 2D2 forward 5'-CCCGGGCAAGGCTCAGCCATGCTCCTG-3' and 2D2 reverse 5'-GCGGCCGCAATTCCCAGAGACATCCCTCC-3'. Food and water were provided *ad libitum*. Breeding pairs and progeny were maintained under RM3 diet (Lillico) at the exception of the progeny of 2D2*Nrf2*$^{+/-}$ x *Nrf2*$^{+/-}$ and *Nrf2*$^{+/-}$ x *Nrf2*$^{+/-}$ that was kept under AIN-76A diet after weaning (Harlan Teklad and TestDiet), as previously described[64]. Mice were used at 6 to 12 weeks of age and littermates were used as controls.

Reagents. Murine MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK; Biopolymers Laboratory of Harvard Medical School and Caslo Laboratory) and OVA (Grade V; Sigma) were dissolved in PBS at 2 mg/ml and 10 mg/ml, respectively. CFA was prepared by grinding and re-suspending *Mycobacterium tuberculosis HR37* (4 mg/ml) in incomplete Freund's adjuvant (Difco; BD Biosciences). PTx (List Biological Laboratories) was dissolved in PBS (100 ng/μl). Phorbol 12-myristate 13-acetate (PMA; Sigma) and inonomycin (Calbiochem) were dissolved in Dimethyl sulfoxide (DMSO; 2.5 mg/ml). Brefeldin A (Epicenter technologies) was dissolved in pure ethanol (1 mg/ml). Anti-CD3 (145-2e11) antibody and IL-23 were purchased from eBioscience. Anti-IL12/23p40 neutralizing antibody (C17.15; kind gift from Dr. Giorgio Trinchieri, National Cancer Institute) and rat isotype IgG2a controls (YKIX-302; anti-canine CD4; a kind donation from Prof. Herman Waldmann, Oxford

University) were produced *in house* from hybridoma cell culture supernatants and purified on a Protein G column.

EAE. *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice or *CD11c-Cre/Hmox1*$^{\Delta/\Delta}$ and *Hmox1*$^{lox/lox}$ were immunized with MOG_{35-55} (100 μg) emulsified in CFA (100 μl) subcutaneously on each side of the belly and received PTx intravenously (200 ng in PBS; 100 μl) after immunization (i.e. 4h and 2 days). 2D2*Nrf2*$^{+/+}$ and 2D2*Nrf2*$^{-/-}$ mice received PTx intravenously (200 ng in PBS; 100 μl) twice at 48h interval. Clinical signs of EAE were evaluated daily for 40 days and scored as follows. For classical EAE: 0 - normal; 1 - limp tail; 2 - partial paralysis of the hind limbs; 3 - complete paralysis of the hind limbs; 4 - hind-limb paralysis and forelimb weakness; 5 - moribund or deceased. For axial-rotatory atypical EAE: 0 - normal; 1 - head turned slightly (ataxia, no tail paralysis); 2 - head turned more pronounced; 3 - inability to walk on a straight line; 4 - laying on side; 5 - rolling continuously unless supported, moribund or deceased.

Adoptive transfer: MOG_{35-55}-immunized 2D2*Nrf2*$^{+/+}$ and 2D2*Nrf2*$^{-/-}$ mice (s.c.; 200 μg in CFA; 100 μl on each side of the belly) received PTx (i.p.; 200 ng in PBS; 100 μl) at the time of immunization and leukocytes were isolated from spleen and draining LN (inguinal and aortic), 7 days after immunization. Single-cell suspensions ($8x10^6$ cells/ml) in supplemented RPMI 1640 (100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS, 50 μM β-mercaptoethanol, 10 mM HEPES and 1 mM sodium pyruvate; 2 mM L-glutamine; Invitrogen and Sigma) were re-stimulated with MOG_{35-55} (20 μg/ml) and recombinant mouse IL-23 (20 ng/ml) for 48h (37°C; 5% CO_2, 95% humidity). Cells were washed in PBS and transferred ($1.5x10^7$ cells/mouse; i.p in 500 μl of PBS) into 10-12 week-old gender-matched wild type, *Nrf2*$^{-/-}$ or *Ifngr1*$^{-/-}$ mice, 20h after sub-lethal irradiation (550 rad; Gammacell 2000; Mølsgaard Medical). EAE was monitored for 40 days and scored as described above.

IL-12/23p40 neutralization *in vivo*. 2D2*Nrf2*$^{+/+}$ and 2D2*Nrf2*$^{-/-}$ mice received PTx intravenously (200 ng in PBS; 100 μl) twice with a 48h interval. At day -1 and 6 post-PTx administration, mice received either anti-IL12/23p40 neutralizing antibody (C17.15, 1 mg/mouse) or a rat isotype-matched IgG2a control antibody (YKIX-302; 1mg/mouse) i.p. dissolved in PBS.

Antigen re-stimulation *in vitro*. Draining LN (inguinal and popliteal) cells were harvested 8 days after footpad immunization (50 μg/per paw of MOG_{35-55} or OVA emulsified in CFA) and homogenized into single-cell suspensions. Cells were plated in 96-well flat bottom microtiter plates ($2.5x10^5$ cells/well) in supplemented RPMI 1640 (200 μl) and re-stimulated with MOG_{35-55} (1, 10 and 100 μg/ml) or OVA (10, 50 and 250 μg/ml) for 72 hours (37°C; 5% CO_2, 95% humidity). Supernatants were analyzed by ELISA for detection of IFN-γ, IL-17A, TNF, IL-10, GM-CSF and IL-13 according to the manufacturer's instructions (eBioscience).

Cell proliferation. Draining LN (inguinal and popliteal) cells were isolated 8 days after footpad immunization (50 μg of MOG_{35-55} or 100 μg of OVA emulsified in CFA per paw). Cells were plated in 96-well flat bottom microtiter plates ($2.5×10^5$ cells/well) in 200 μl of supplemented RPMI 1640 (Invitrogen) and exposed to MOG_{35-55} (1, 10 and 100 μg/ml) or OVA (10, 50 and 250 μg/ml) for 72 hours (37°C, 5% CO_2, 95% humidity). For *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ as well as *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ cells, proliferation was assessed by [^{3}H]-thymidine incorporation (1 μCi/well; Perkin Elmer) during the last 6 hours of culture evaluated in a scintillation counter (Tomtec; Pharmacia). 2D2*Nrf2*$^{+/+}$ and 2D2*Nrf2*$^{-/-}$ cell proliferation was assessed by carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) staining. Briefly, cells were re-suspended in HBSS ($2.5x10^6$ cells/ml), incubated with CFSE (5 μM; 5 min.) and stained for flow cytometry. Cell numbers were assessed by flow cytometry, using a fixed number of latex beads (Beckman Coulter) co-acquired with a pre-established volume of the cellular suspensions.

Isolation of splenocytes and LN cells. Spleen and LN cells were harvested and homogenized into a single-cell suspension in PBS 2% FCS. Splenocytes were subjected to erythrocyte lysis in 3 ml of a hypotonic red blood cell lysis buffer (155 mM NH4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA) while centrifuging (375g, 5 min., 4ºC). Cells were washed twice in 15 ml PBS 2% FCS (375 g, 5 min., 4ºC). For intracellular cytokine staining, cells were cultured in supplemented RPMI 1640 containing 50 ng/ml PMA, 500 ng/ml ionomycin and 10 μg/ml brefeldin A for 4h at 37°C (5% CO_2, 95% humidity). Surface and intracellular stainings were analyzed by flow cytometry.

CNS-infiltrating leukocytes. Brain and spinal cord were harvested from transcardially perfused mice (20 ml cold PBS). Single-cell suspensions were prepared in HBSS (Invitrogen) containing Collagenase VIII (0.2 mg/ml; Sigma) by homogenizing the tissue between two glass slides, digesting (30 min.; 37°C; 5% CO_2, 95% humidity) and filtering (100-μm; BD Falcon). After centrifugation (375g, 5 min., 4ºC) in PBS, leukocytes were separated on a discontinuous 30% percoll gradient (Sigma) by centrifugation (1041g, 30 min., RT) erythrocytes were lysed in hypotonic red blood cell lysis buffer (5 min., RT). For intracellular cytokine staining, cells were cultured in supplemented RPMI 1640 with PMA, ionomycin and brefeldin A as described above. Surface and intracellular stainings were analyzed by flow cytometry.

Splenic DC isolation. Spleens were harvested and perfused with Collagenase D (2 mg/ml; Roche) in HBSS, cut into small pieces and incubated at 37°C for 30 min. (5% CO_2, 95% humidity). The single-cell suspensions were filtered (70-μm) and DC in these suspensions were isolated using anti-CD11c positive magnetic selection according to the manufacturer's instructions (Miltenyi Biotech) and sorted as $CD11c^+MHCII^+TCR\beta^-IgM^-CD19^-$ cells using a MoFlo cell sorter (Beckman Coulter). DC purity was routinely higher than 98% (*Fig 4a-d*).

Flt3L-derived bone marrow DC. Bone marrow cells were isolated and cultured (10 days, 37ºC; 5% CO_2, 95% humidity) in supplemented RPMI 1640 medium in 6-well

plates (3-5x10^6 cells/well) with 5% of cell culture supernatant containing murine Flt3L produced by the CHO-Flt3L cell line (kind gift from Dr. Caetano Reis e Sousa, Imperial College, London, UK). The CHO cell line was originally provided as a kind gift by Dr. Nic Nicola (The Walter and Eliza Hall Institute). The CHO-Flt3L cell culture supernatant was produced as follows: murine Flt3L was expressed in CHO cells transfected with a soluble Flt3L/FLAG peptide in the mammalian expression plasmid pEF-BOS[65]. CHO-Flt3L cells were grown in RPMI 1640 media containing 5% FCS and 400 μg/ml geneticin (G418; Sigma) for 7 days. Supernatant was collected, centrifuged (375g, 5 min., 4ºC), filtered (22-μm) and stored at -20ºC until use.

GM-CSF-derived bone marrow DC. Bone marrow cells were isolated and cultured (6 days, 37ºC; 5% CO_2, 95% humidity) in supplemented RPMI 1640 medium with 1% GM-CSF-conditioned medium in 6-well plates (1x10^6 cells/well). Medium was replaced every 48h until day six. GM-CSF-conditioned medium was prepared from the J558 plasmacytoma (kind gift from Dr. Brigitta Stockinger, National Institute for Medical Research) as follows. Cells were cultured in D-MEM: F12 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Sigma), 50 μM β-mercaptoethanol and 1 mg/ml Geneticin (G418; all from Invitrogen and Sigma). Confluent cells were transferred and grown in 50 ml of medium without Geneticin for 3 days. The cell suspension was collected, centrifuged (200g, 10 min., 4ºC) and supernatant filtered through 0.22-μm and stored at -20ºC until use.

DC stimulation. DC were plated in 96-well round bottom microtiter plates (0.5x10^5 (IL12/23p40 and IL-6) or 2.0x10^5 cells/well (IL-12p70 and TNF)) in supplemented RPMI 1640 medium (250 μl) and stimulated with LPS (100 ng/ml; *E.coli* O127:B8; Sigma), Poly(I:C) (50 μg/ml; Invivogen) or CpG (0.1 μM, Invivogen) for qRT-PCR analysis for 1h (*Il23p19*) or 4h (others) and for ELISA 24h. Flt3L-derived bone marrow DC were plated (0.5x10^5 cells/well) in supplemented RPMI 1640 medium (250 μl) and stimulated with LPS (starting at 1.25x10^4 ng/ml and diluted sequentially

1:5; *E.coli* O127:B8; Sigma), Poly(I:C) (starting at 50 μg/ml and diluted sequentially 1:5; Invivogen) or CpG (starting at 2 μM and diluted sequentially 1:5; Invivogen). Cells were incubated for 24h (37°C; 5% CO_2, 95% humidity) and supernatants analyzed by ELISA for IL-12/23p40 (BD biosciences) according to manufacturer's instructions.

$CD4^+$ T cell purification and antigen-presenting assays. Spleen and LN cells were homogenized into single-cell suspensions, as described above. Naïve $CD4^+$ T cells were sorted from 2D2*Nrf2*$^{+/+}$ or 2D2*Nrf2*$^{-/-}$ as $CD4^+CD62L^{high}CD25^-CD11c^-$ cells in the presence of EDTA to disrupt DC-T cell complexes (MoFlo cell sorter; Beckman Coulter). Purity was routinely higher than 98%. For antigen-presenting assays, splenic *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ DC were plated in 96-well round bottom microtiter plates ($1x10^4$ cells/well) in supplemented RPMI 1640 and pulsed with MOG_{35-55} (100 ng/ml) for 1h in presence of LPS (100 ng/ml) (37 °C; 5% CO_2, 95% humidity). After centrifugation (666g, 2 min., 4ºC), supernatants were removed and T cells ($1x10^5$ cells/well) added (250 μl) in supplemented RPMI 1640 medium with LPS (100 ng/ml). Cells were cultured for 96h (37°C; 5% CO_2, 95% humidity). After centrifugation (666g, 2 min., 4ºC) cells were re-stimulated (48h) with plate-bound anti-CD3 mAb (2.5 μg/ml; PBS; 1h; 37°C). Anti-IL-12/23p40 neutralizing mAb (C17.15; 20 μg/ml) was used to suppress IL-12/23p40 activity during DC pulsing and in the following 96h of co-culture with T cells. Rat IgG2a (YKIX-302; 20 μg/ml) mAb was used as control. Supernatants were analyzed by ELISA for IFN-γ, IL-17A, TNF, IL-10, GM-CSF, IL-13 (all from eBioscience), IL-2 and IL-4 (BD Biosciences) according to the manufacturer's instructions.

Flow cytometry and antibodies. The following mAb were used: FcγIII/II receptor (2.4G2), anti-CD4 (GK1.5-8 and RM4-5), anti-CD11b (M1/70), anti-CD25 (PC 61), anti-IgM (R33.24.12), anti-TCRβ (H57-597). anti-CD11c (HL3), anti-CD40 (3/23), anti-CD45 (30-F11), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-Ly6G (RB6-8C5), anti-CD19 (MB19-1), anti-CD62L (Mel 14) (prepared *in house* from hybridoma culture supernatants), anti-MHC class II (AF6-120.1; BD Biosciences and M5/114;

eBioscience) and anti-F4/80 (CI:A3-1; Serotec). The following antibodies were used to sort DC, anti-CD11c (HL3), anti-MHC class II (M5/114), anti-TCRβ (H57-597), anti-CD19 (MB19-1) and anti-IgM (R33.24.12), and naïve T cells were sorted with the antibodies against CD4 (GK1.5-8), CD25 (PC 61), CD62L (Mel 14) and CD11c (HL3). Antibodies were directly conjugated to FITC, Alexa488, PE, PerCP, APC, Cy5, Alexa467 or APC-Cy5.

For surface staining (flow cytometry and sorting), cells were incubated with anti-FcγIII/II receptor antibody (2.4G2) in PBS 2% FCS (20 min., 4°C). Surface staining was performed in PBS 2% FCS (20 min., 4°C). After washing and centrifugation (666g, 2 min., 4ºC) cells were incubated with streptavidin-allophycocyanin for (20 min., 4°C) to detect biotinylated antibodies. For flow cytometry analysis of surface stainings dead cells were excluded using propidium iodide (1.7 μg/ml).

For intracellular cytokine staining cells were fixed (2% paraformaldehyde; 30 min., RT) and permeabilized (0.5% saponin in PBS 2% FCS, i.e. permeabilization buffer)(10 min., RT). Cells were incubated with anti-IFN-γ (XMG1.2; BD Biosciences) or anti-IL-17 (eBioTC11-18H10.1; eBioscience) mAb in permeabilization buffer (20 min., 4°C). For intracellular Foxp3 staining, cells were stained with anti-mouse Foxp3 conjugated (FJK-16s; eBioscience) with PE using the Foxp3 detection set (eBioscience) according to the manufacturer's instructions.

Cell numbers were assessed by flow cytometry, using a fixed number of latex beads (Beckman Coulter) co-acquired with a pre-established volume of the cellular suspensions.

Flow cytometry detection was done in the FACSCalibur (BD Biosciences), using the cellquest software (BD Biosciences) for acquisition. Post-acquisition analysis was performed with FloJo software (Treestar).

RNA isolation and qRT-PCR. For mRNA analysis of Nrf2-dependent genes in the CNS, spinal cords from individual mice were harvested, snap-frozen in liquid nitrogen, grinded and resuspended in TRIzol (Invitrogen). RNA was extracted with chloroform and purified with the RNeasy Mini Kit (Qiagen). For mRNA analysis of

Nrf2-dependent genes in splenic DC, cells from at least 3 spleens were pooled, sorted, stimulated *in vitro* and RNA was extracted with the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed from 2 μg (spinal cord) or 0.3-0.5 μg (DC) of RNA using random hexamer primers (0.3 μg/reaction; Invitrogen), dNTPs (0.5 mM/reaction; Invitrogen)(5 min., 65°C). 5x First Strand buffer (Invitrogen) was added in the presence of DTT (10 mM/reaction; Invitrogen) and RNAse Out recombinant ribonuclease inhibitor (40 U/reaction; Invitrogen)(2 min., 42°C). SuperScriptII reverse transcriptase (200 U/reaction; Invitrogen) was added completing a final volume of 20 μl (50 min., 42°C; 15 min., 70°C). cDNA was diluted 1:25 (spinal cord) or 1:5 (DC) and (1 μl) used for PCR reactions (10 μl) using the Power SYBRGreen PCR master mix (Applied Biosystems) and optimal primer concentrations (previously determined for each transcript). PCR products were detected by qRT-PCR (ABI-7900HT; Applied Biosystems)(2 min., 50°C, 10 min., 95°C, and 40 cycles of 15 sec at 95°C, 1 min., 60°C). Primers used to amplify mouse mRNA transcripts were designed using the Primer3 software (Whitehead Institute for Biomedical Research, Steve Rozen and Helen Skaletsky) according to the specifications of the ABI-7900HT equipment (Applied Biosystems) and are listed below: *Nrf2* 5'-TAGATGACCATGAGTCGCTTGCCC-3' and 5'-ATAGCTCCTGCCAAACTTGCTCC-3', *Nqo1* 5'-CCGAACACAAGAAGCTGGAAGCTG-3' and 5'-AGGCAAATCCTGCTACGAGCAC-3', *Hmox1* 5'-AAGGAGGTACACATCCAAGCCGAG-3' and 5'-GATATGGTACAAGGAAGCCATCACCAG-3', *Fth1* 5'-ATGCCGAGAAACTGATGAAGCTGC-3' and 5'-TGCACACTCCATTGCATTCAGCC-3', *Gstt1* 5'-AGCCATTCTCAACTACATCGCCAC-3' and 5'-GGGGGACATAATACCAATTGCCCAATC-3', *Gpx* 5'-TCGGTTTCCCGTGCAATCAGTTC-3' and 5'-CCTTCTCACCATTCACTTCGCACTTC-3', *Gsr* 5'-ACCATGATTCCAGATGTTGACTGCC-3' and 5'-CCCTTTTCATCCGTCTGAATGCCC-3', *Mt1* 5'-ACGTGCTGTGCCTGATGTGACGAACAG-3' and 5'-TAGACTCAAACAGGCTTTTATTATTAACG-3', *Mt2* 5'-CAACTGCTCCTGTGCCTCCG-3' and 5'-ACGGCTTTTATTGTCAGTTACATGCTTTAT-3', *Prdx1* 5'-CAAGGAGGATTGGGACCCATGAAC-3' and 5'-GCCCCTGAAAGAGATACCTTCATCAGC-3', *Cat* 5'-ACCCCTATTGCCGTTCGATTCTCC-3' and 5'-TTGTTTCCCACAAGATCCCAGTTACC-3', *Sod2* 5'-TAAGGGTGGTGGAGAACCCAAAGGAG-3' and 5'-

TTATTGAAGCCAAGCCAGCCCCAG-3', *Trn1* 5'-GGACCTTGCAAAATGATCAAGCCCTTC-3' and 5'-AGCAACATCCTGGCAGTCATCCAC-3', *Gclm* 5'-GCCACCAGATTTGACTGCCTTTG-3' and 5'-TGCTCTTCACGATGACCGAGTACC-3', *Gclc* 5'-ACATCTACCACGCAGTCAAGGACC-3' *and* 5'-CTCAAGAACATCGCCTCCATTCAG-3', *Cd36* 5'-GATGACGTGGCAAAGAACAG-3' and 5'-TCCTCGGGGTCCTGAGTTAT-3', *Il12p35* 5'-CCACCCTTGCCCTCCTAAAC-3' and 5'-GTTTTTCTCTGGCCGTCTTCA-3', *Il12p40* 5'-GGAAGCACGGCAGCAGAATA-3' and 5'-AACTTGAGGGAGAAGTAGGAATGG-3', *Il23p19* 5'-TGCTGGATTGCAGAGCAGTAA-3' and 5'-GCATGCAGAGAT TCCGAGAGA-3' and Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh)* 5'-AACTTTGGCATTGTGGAAGG-3' and 5'-ACACATTGGGGGTAGGAACA-3'. All other transcripts were analyzed using a TaqMan Gene Signature Mouse Immune Array (Applied Biosystems) according to the manufacturer's instructions. Transcript number was calculated from the Ct of each gene using a $2^{-\Delta\Delta CT}$ method (relative number) and normalizing results to *Gapdh*.

Statistics. EAE severity was analyzed daily with the Nonparametric Mann-Whitney *U* test. Disease incidence was analyzed by the Fisher test and survival by Log-rank analysis. Statistical analysis of IL-12/23p40 levels in Flt3L-derived bone marrow DC was performed by linear regression analysis where the dependent and independent variables are the concentration of IL-12/23p40 and the dose of stimulus, respectively. All other statistical analysis were performed using the Nonparametric Mann-Whitney *U* test, when sample size was smaller than 5, or did not follow normal distribution, and when it did Unpaired Student's *t*-test for unequal variances was used. Normal distributions were assessed using the Kolmogorov-Smirnov test. $p<0.05$ was considered statistically significant.

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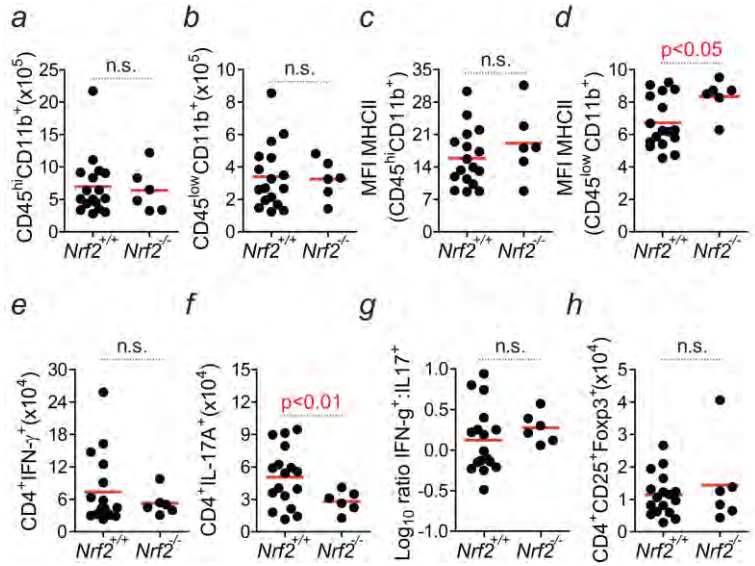
8. Supplementary Figures

Gene	1/ Average ΔCt x 100
Nrf2	8.81
Nqo1	9.51
Hmox1	8.60
Fth	242.49
Prdx1	11.99
Cat	12.02
Sod2	20.58
Gstt1	5.29
Gpx	17.63
Gsr	9.41
Mt1	7.89
Mt2	10.88
Trn1	11.36
Gclm	17.07
Gclc	10.81
Cd36	8.54

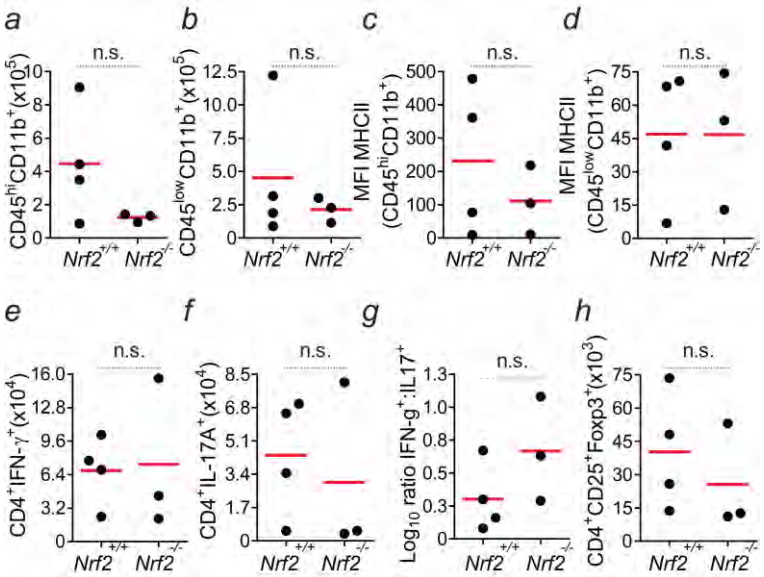
Supplementary Table 1. Relative level of expression of Nrf2-dependent genes in naïve wild type mice. Data is shown as the inverse of the average ΔCt multiplied by a factor of 100 (1/ average ΔCtx100). ΔCt is the difference between the threshold cycle (Ct) of the gene of interest and the Ct of *Gapdh.* Data was pooled from at least three independent experiments.

Genotype	*n*	Mortality (%)
$Nrf2^{+/+}$	17	0 (0/17)
$Nrf2^{-/-}$	16	6,25 (1/16)

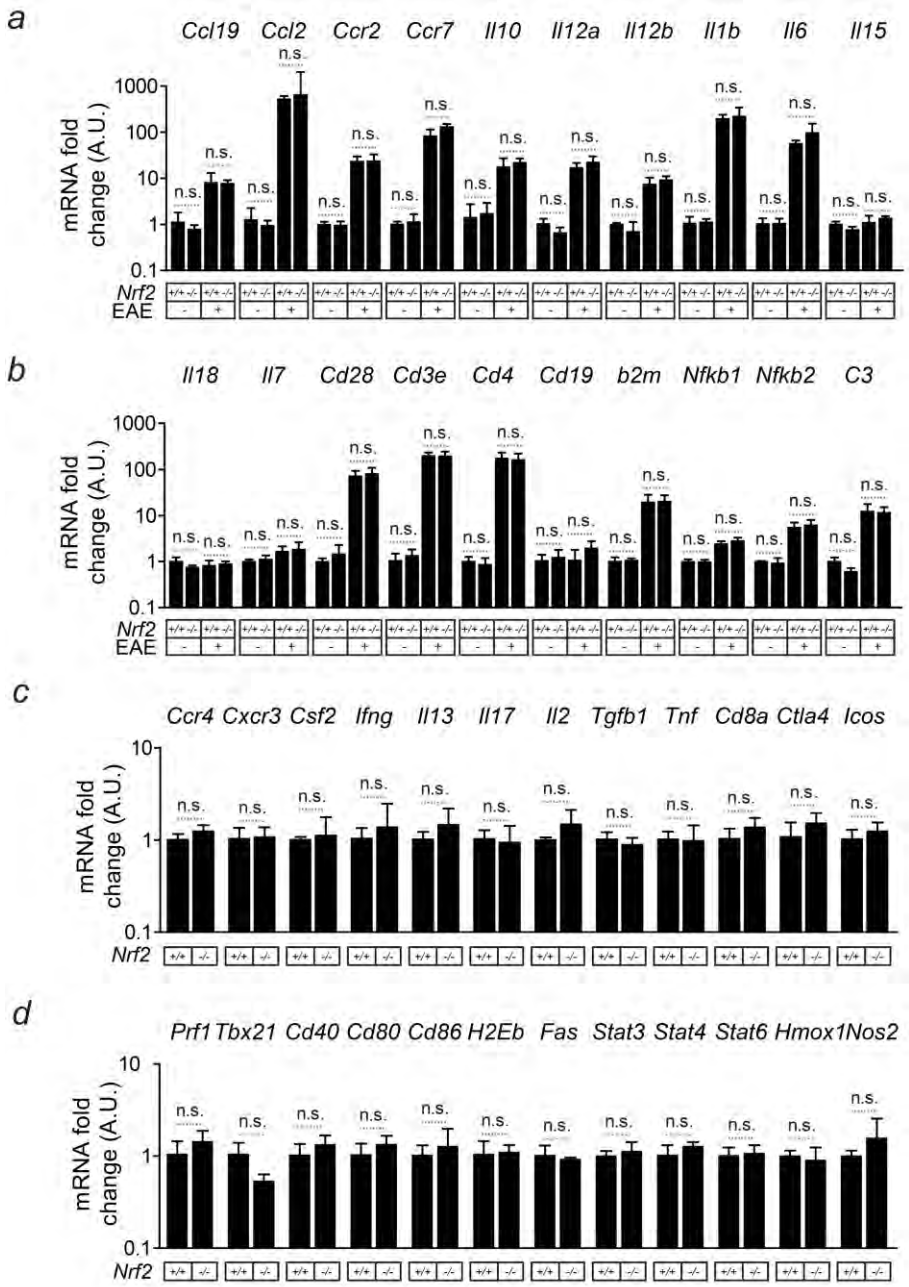
Supplementary Table 2. Mortality associated with OVA plus CFA immunization in EAE induction. $Nrf2^{+/+}$ (n=17) and $Nrf2^{-/-}$ (n=16) mice were immunized with OVA (100 μg) emulsified in CFA (100 μl) subcutaneously on each side of the belly and received PTx intravenously (200 ng in PBS; 100 μl) after immunization (i.e. 4h and 2 days). Data is shown as the percentage of mice that died over the course of 40 days post-immunization and was pooled from three independent experiments.



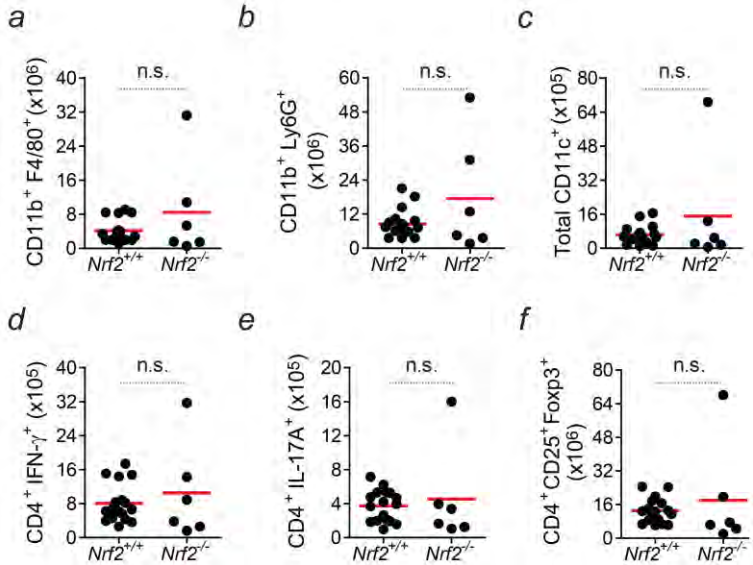
Supplementary Figure 1. Nrf2 does not modulate leukocyte infiltration into the CNS during EAE. Flow cytometry analysis of brain and spinal cord leukocyte infiltrates in MOG_{35-55}-immunized *Nrf2*$^{+/+}$ (n=17) and *Nrf2*$^{-/-}$ (n=6) mice receiving PTx, analyzed at the onset of EAE (day 13). Number of (***a***) macrophages ($CD45^{hi}CD11b^{+}$) and (***b***) microglia ($CD45^{low}CD11b^{+}$). Mean intensity of fluorescence (MFI) of MHC class II expression in (***c***) macrophages and (***d***) microglia. Number of (***e***) T_H1, (***f***) T_H17 and (***h***) T_{REG} cells ($CD4^{+}CD25^{+}Foxp3^{+}$). (***g***) Ratio of T_H1/T_H17 cells calculated as Log_{10} (number $CD4^{+}IFN\text{-}\gamma^{+}$/number $CD4^{+}IL\text{-}17A^{+}$). Circles correspond to single mice and lines to the average of the group. Data was pooled from two independent experiments.



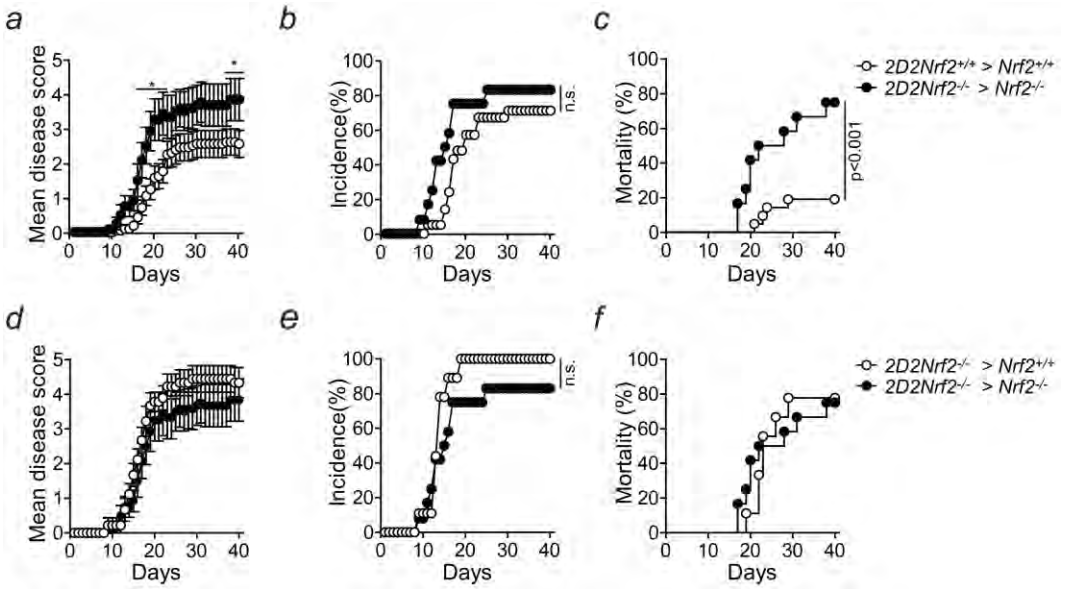
Supplementary Figure 2. Nrf2 does not modulate leukocyte infiltration into the CNS during EAE. Flow cytometry analysis of brain and spinal cord leukocyte infiltrates in 2D2*Nrf2*$^{+/+}$ (n=4) and 2D2*Nrf2*$^{-/-}$ (n=3) mice displaying symptoms of EAE (day 22 post-PTx administration). Number of (***a***) of macrophages ($CD45^{hi}CD11b^{+}$) and (***b***) microglia ($CD45^{low}CD11b^{+}$). MFI of MHC class II expression in (***c***) macrophages and (***d***) microglia. Number of (***e***) T_H1, (***f***) T_H17 and (***h***) T_{REG} cells. (***g***) Ratio of T_H1/T_H17 cells calculated as Log_{10} (number $CD4^{+}IFN\text{-}\gamma^{+}$/number $CD4^{+}IL\text{-}17A^{+}$). Circles correspond to single mice and lines to the average of the group. Data was pooled from two independent experiments.



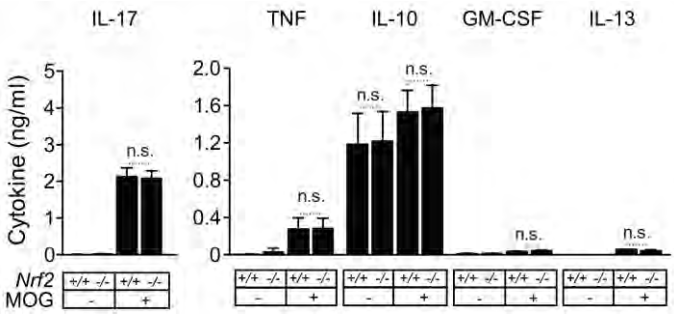
Supplementary Figure 3. Expression of Nrf2 does not modulate the expression of immune-related genes in the CNS during EAE. Transcripts were quantified by qRT-PCR in the spinal cord of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice, before and at the onset of EAE (day 13). (***a, b***) Data is shown as the mean of the fold change in mRNA expression relative to wild type naïve mice ± STD. (***c, d***) Data is shown as mean fold change of mRNA expression relative to wild type mice undergoing EAE ± STD. Data was pooled from 4 independent experiments (n=12 pooled in sets of 3 mice).



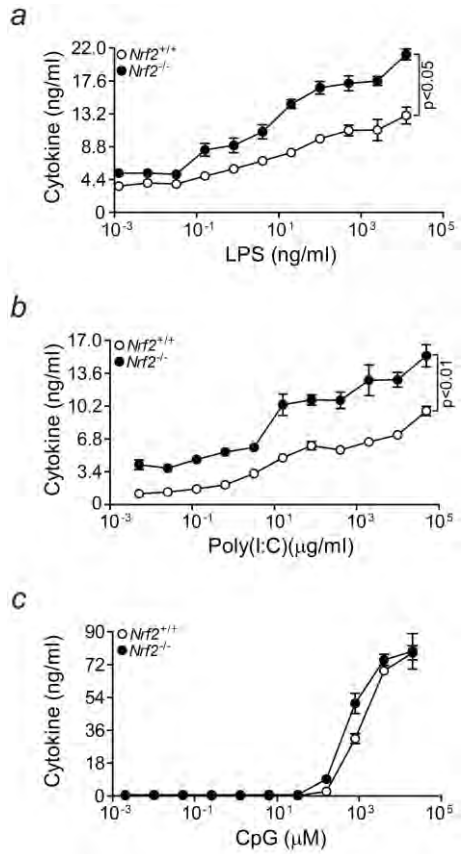
Supplementary Figure 4. Nrf2 does not modulate macrophage or T_H cell activation in the spleen during EAE. Flow cytometry analysis of the splenic populations in *Nrf2*$^{+/+}$ (n=17) and *Nrf2*$^{-/-}$ (n=6) mice at the onset of EAE (day 13). (***a***) Total number of macrophages, (***b***) neutrophils, (***c***) dendritic cells, (***d***) T_H1, (**e**), T_H17 and (***f***) T_{REG} cells. Each data point corresponds to a mouse and the line is the average of the group. Data is shown as the pool of two independent experiments**.**



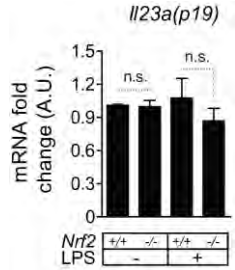
Supplementary Figure 5. Nrf2 inhibits leukocyte encephalitogenicity. (***a***) Mean EAE score ± SEM of *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ mice adoptively transferred with leukocytes from MOG_{35-55}-immunized *2D2Nrf2*$^{+/+}$ (white; n=21) or *2D2Nrf2*$^{-/-}$ leukocytes (black; n=12) mice, respectively. (***b***) Disease incidence in the same mice as (a). (***c***) Mortality in the same mice as (a). (***d***) Mean EAE score ± SEM in *Nrf2*$^{+/+}$ (white, n=9) or *Nrf2*$^{-/-}$ mice (black; n=12) adoptively transferred with leukocytes from MOG_{35-55}-immunized *2D2Nrf2*$^{-/-}$ mice. (**e**) Disease incidence in the same mice as (d). (***f***) Mortality in the same mice as (d). Data was pooled from five independent experiments with similar results. The mice are the same as in Figure 2. (*) p<0.05.



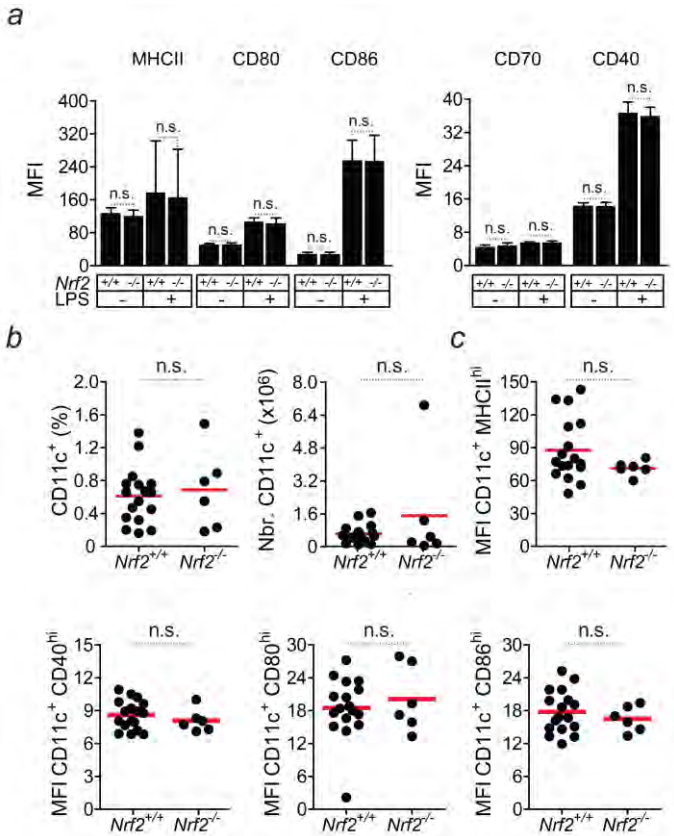
Supplementary Figure 6. Nrf2 does not modulate the expression of IL-17A, TNF, IL-10, GM-CSF and IL-13 in C57BL/6 mice. Concentration of IL-17A, TNF, IL-10, GM-CSF and IL-13 in supernatants from leukocytes isolated from MOG_{35-55}-immunized *Nrf2*$^{+/+}$ (n=9) or *Nrf2*$^{-/-}$ (n=7) mice and re-challenged *in vitro* with MOG_{35-55} (100 μg/ml; 72h). Data is shown as mean ± SEM from three independent experiments with similar trend (n=2-3 mice/group), each performed in triplicate.



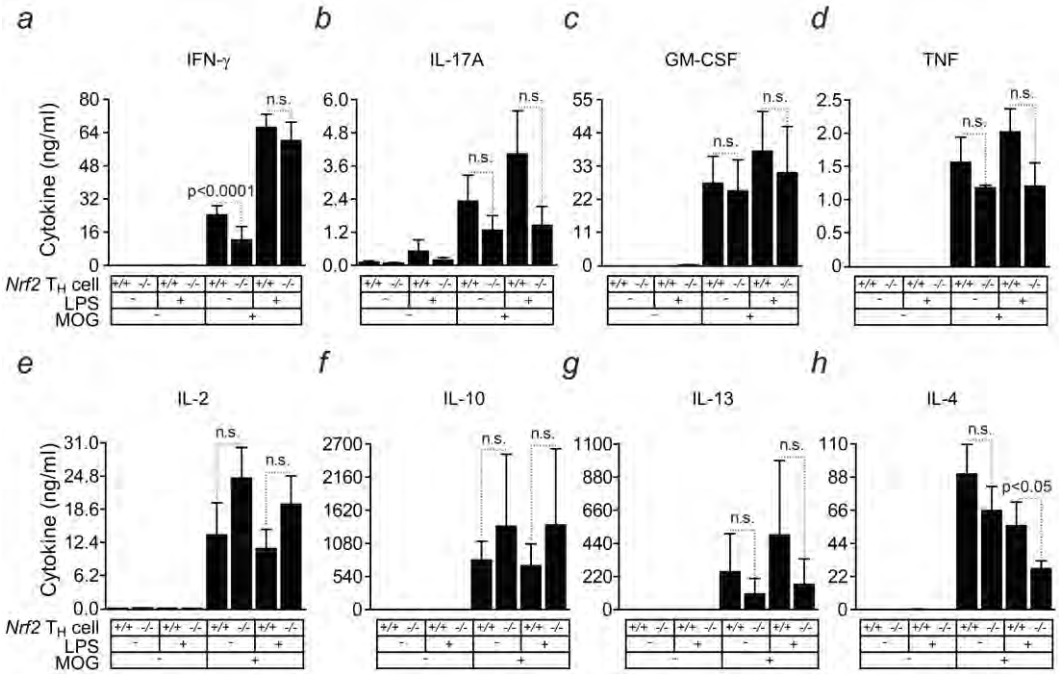
Supplementary Figure 7. Nrf2 inhibits the production of IL-12/23p40 in Flt3L-derived bone marrow DC. Concentration of IL-12/23p40 in cell culture supernatants from Flt3L-derived DC generated from the bone marrow of *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ C57BL/6 mice stimulated with (***a***) LPS, (***b***) Poly(I:C) and (***c***) CpG. Data is shown as mean ± STD from one experiment representative of two independent experiments, performed in triplicate.



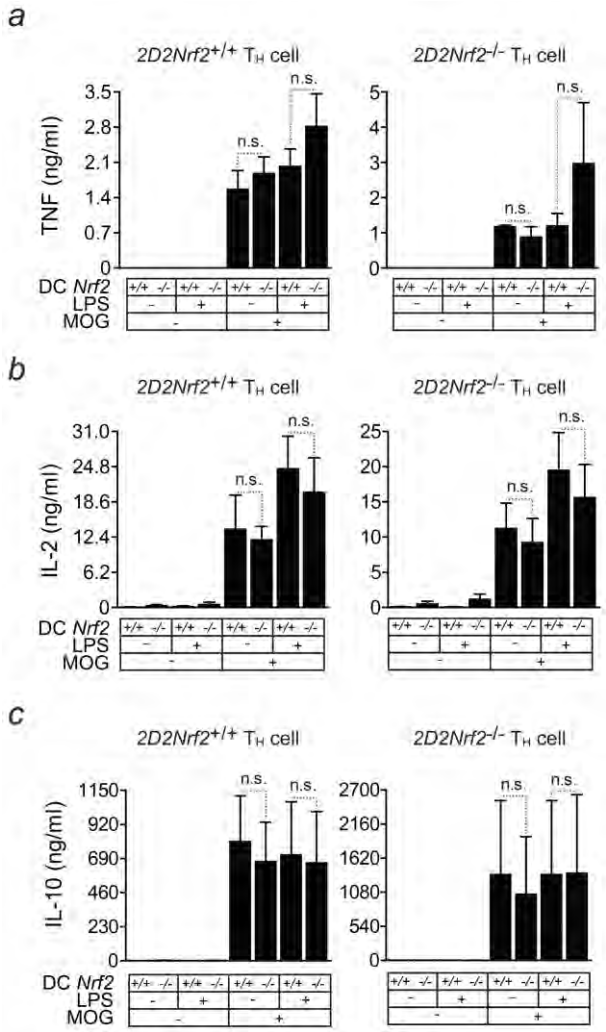
Supplementary Figure 8. Nrf2 expression in DC does not affect IL-23p19 production. mRNA expression of *Il23p19* analyzed by qRT-PCR in splenic DC from *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ C57BL/6 mice. When indicated (+) DC were stimulated *in vitro* with LPS (100 ng/ml; 1h). Data is shown as mean fold change of mRNA expression relative to wild type unstimulated cells ± STD from two independent experiments, with similar trend.



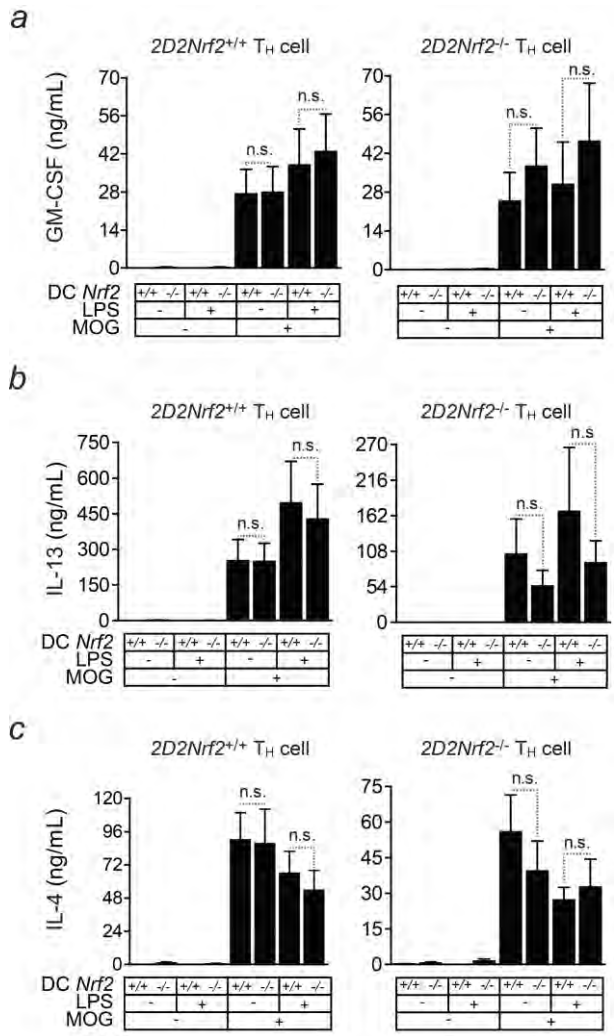
Supplementary Figure 9. Nrf2 does not modulate the expression of MHC class II or costimulatory molecules in DC. (***a***) MFI of MHC class II, CD80, CD86, CD70 and CD40 in splenic DC (CD11c^{+}) from *Nrf2*$^{+/+}$ (n=6) and *Nrf2*$^{-/-}$ (n=6) mice, receiving or not LPS *in vivo* (10 μg/mouse; 6h). Data is shown as mean ± STD from individual mice (n=6) pooled from two independent experiments. (***b***) Percentage and total number of CD11c^{+} cells in the spleen of *Nrf2*$^{+/+}$ (n=6) and *Nrf2*$^{-/-}$ (n=6) mice at the onset of EAE (day 13) (***c***) MFI of MHC class II, CD40, CD80 and CD86, gated in CD11c^{+} cells in the spleen of *Nrf2*$^{+/+}$ (n=6) and *Nrf2*$^{-/-}$ (n=6) mice at the onset of EAE (day 13). Each data point corresponds to a mouse and the line is the average of the group. Data is shown from two pooled independent experiments.



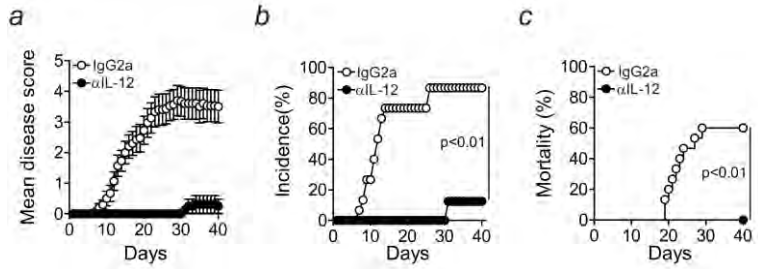
Supplementary Figure 10. Effect of Nrf2 expression on cytokine production by T_H cells. Concentration of (***a***) IFN-γ, (***b***) IL-17A, (***c***) GM-CSF, (***d***) TNF, (***e***) IL-2, (***f***) IL-10, (***g***) IL-13 and (***h***) IL-4 by T_H cells from naïve 2D2*Nrf2*$^{+/+}$ or 2D2*Nrf2*$^{-/-}$ mice, co-cultured with *Nrf2*$^{+/+}$ MOG_{35-55}-pulsed (100 μg/ml) splenic DC (96h). When indicated (+) DC were stimulated with LPS (100 ng/ml). T_H cells were re-stimulated with an anti-CD3 mAb (2.5 μg/ml) and cytokines were measured in cell culture supernatants by ELISA. Data is shown as mean ± SEM from at least three independent experiments, each performed in quintuplicate.



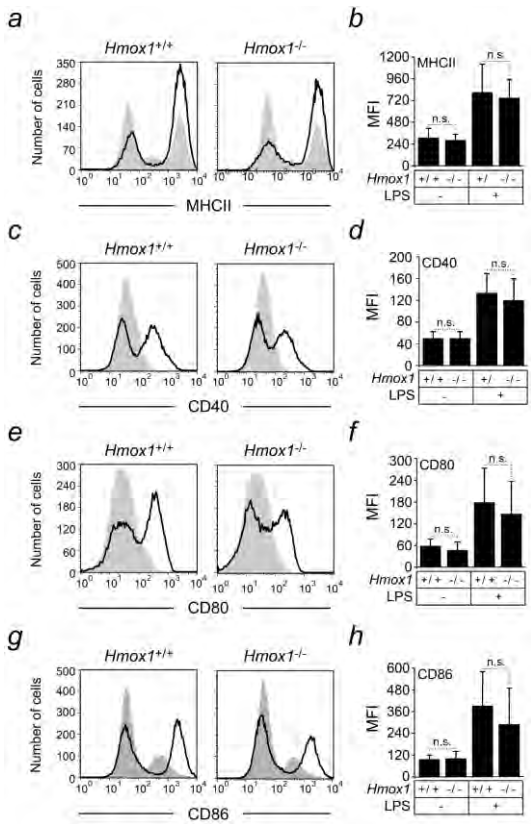
Supplementary Figure 11. Nrf2 expression in DC does not affect TNF, IL-2, and IL-10 production by T_H cells. Concentration of (***a***) TNF, (***b***) IL-2 and (***c***) IL-10 in cell culture supernatants of $CD4^+$ T_H cells from naïve 2D2*Nrf2*$^{+/+}$ or 2D2*Nrf2*$^{-/-}$ mice co-cultured with MOG_{35-55}-pulsed splenic DC from *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ mice. When indicated (+), DC were stimulated with LPS (100 ng/ml). Cytokine production was assessed 48h after re-stimulation with an anti-CD3 (2.5 μg/ml) mAb. Data is shown as mean ± SEM pooled from at least three independent experiments, each performed at least in quintuplicate.



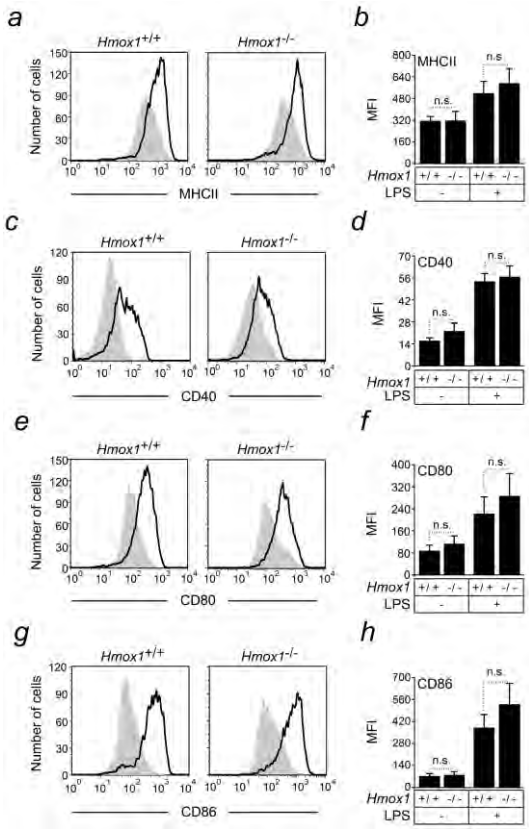
Supplementary Figure 12. Nrf2 expression in DC does not affect GM-CSF, IL-13 or IL-4 production by T_H cells. Concentration of (***a***) GM-CSF, (***b***) IL-13 and (***c***) IL-4 in cell culture supernatants of $CD4^+$ T_H cells from naïve 2D2*Nrf2*$^{+/+}$ or 2D2*Nrf2*$^{-/-}$ mice co-cultured with MOG_{35-55}-pulsed splenic DC from *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ mice (96h). When indicated (+), DC were stimulated with LPS (100 ng/ml). Cytokine production was assessed 48h after re-stimulation with an anti-CD3 (2.5 μg/ml) mAb. Data is shown as mean ± SEM pooled from at least three independent experiments, each performed at least in quintuplicate.



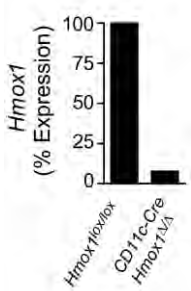
Supplementary Figure 13. EAE induced by PTx in 2D2 mice is mediated by IL-12/23p40 production. (***a***) Mean EAE score ± SEM in 2D2*Nrf2*$^{+/+}$ treated with an anti-IL-12/23p40 neutralizing antibody (αIL-12; 20 μg/ml; black; n=8) *vs.* an isotype-matched control antibody (IgG2a; 20 μg/ml; white; n=15). Data is pooled from three independent experiments with similar trend. (***b***) Disease incidence in the same mice as (a). (***c***) Mortality in the same mice as (a).



Supplementary Figure 14. Expression of *Hmox1* does not modulate the maturation of GM-CSF-derived bone marrow DC *in vitro*. Histogram comparing surface expression of (***a***) MHC class II, (***c***) CD40, (***e***) CD80 and (***g***) CD86 in GM-CSF-derived CD11c^{+} DC generated from the bone marrow of *Hmox1*$^{+/+}$ (left) and *Hmox1*$^{-/-}$ (right) mice, stimulated (back line) or not (grey) with LPS *in vitro* (100 ng/ml; 24h). Each line represents cells from a single mouse, in one out of 3 independent experiments with similar results (n=1-4 mice/group). MFI of (***b***) MHC class II, (***d***) CD40, (***f***) CD80 and (***h***) CD86 in GM-CSF-derived CD11c^{+} DC generated from the bone marrow of *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ mice, stimulated (+) or not with LPS *in vitro* (100 ng/ml; 24h). Data is shown as mean ± SEM from three pooled independent experiments, with similar results.



Supplementary Figure 15. Expression of *Hmox1* does not modulate the maturation of splenic DC *in vivo*. Histogram comparing surface expression of (***a***) MHC class II, (***c***) CD40, (***e***) CD80 and (***g***) CD86 in splenic DC ($CD11c^+$) from severe combined immunodeficiency (*SCID).Hmox1*$^{+/+}$ (left) and *SCID.Hmox1*$^{-/-}$ (right) mice, stimulated (open black line) or not (filled grey) with LPS *in vivo* (10 μg/mouse; 6h). Each line represents cells from a single mouse, in one out of two independent experiments with similar results. MFI of (***b***) MHC class II, (***d***) CD40, (***f***) CD80 and (***h***) CD86 in splenic $CD11c^+$ DC from *SCID.Hmox1*$^{+/+}$ (n=5) and *SCID.Hmox1*$^{-/-}$ (n=3) mice, stimulated (+) or not with LPS *in vivo* (10 μg/mouse; 6h). Data is shown as mean ± STD from two independent experiments with similar results (n=2-3 mice/experiment).



Supplementary Figure 16. DC from *CD11c-Cre/Hmox1*$^{\Delta/\Delta}$ mice do not express *Hmox1*. *Hmox1* mRNA analyzed by qRT-PCR in sorted splenic DC from *Hmox1*$^{lox/lox}$ (n=2 pooled) *vs. CD11c-Cre/Hmox1*$^{\Delta/\Delta}$ (n=2 pooled) C57BL/6 mice with EAE (after day 40). Data is shown as percentage of the mean fold change of mRNA expression relative to *Hmox1*$^{LoxP/LoxP}$ DC from one experiment.

Chapter 3

Heme Oxygenase-1 and Carbon Monoxide Suppress Autoimmune Neuroinflammation

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1. Abstract

Heme oxygenase-1 (HO-1, encoded by *HMOX1*) dampens inflammatory reactions via the catabolism of heme into CO, Fe, and biliverdin. We report that expression of HO-1 dictates the pathologic outcome of experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS). Induction of EAE in *Hmox1*$^{-/-}$ C57BL/6 mice led to enhanced CNS demyelination, paralysis, and mortality, as compared with *Hmox1*$^{+/+}$ mice. Induction of HO-1 by cobalt protoporphyrin IX (CoPPIX) administration after EAE onset reversed paralysis in C57BL/6 and SJL/J mice and disease relapse in SJL/J mice. These effects were not observed using zinc protoporphyrin IX, which does not induce HO-1. CoPPIX protection was abrogated in *Hmox1*$^{-/-}$ C57BL/6 mice, indicating that CoPPIX acts via HO-1 to suppress EAE progression. The protective effect of HO-1 was associated with inhibition of MHC class II expression by APC and inhibition of T_H and CD8 T cell accumulation, proliferation, and effector function within the CNS. Exogenous CO mimicked these effects, suggesting that CO contributes to the protective action of HO-1. In conclusion, HO-1 or exposure to its end product CO counters autoimmune neuroinflammation and thus might be used therapeutically to treat MS.

2. Introduction

MS is a presumed autoimmune disorder that targets the CNS[1]. Neuroinflammatory lesions associated with MS progression are triggered upon interaction of activated pathogenic T_H cells with APC within the CNS. This leads to the generation of a pro-inflammatory response that causes irreversible oligodendrocyte injury, neuron demyelination, and eventually axonal loss, the main pathologic hallmarks of MS[2-4].

The clinical course of MS is, in most cases, associated with transitory episodes of remission[2], suggesting that regulatory mechanisms must operate to counter neuroinflammation and/or to promote neuron regeneration. Such mechanisms may involve, but are most probably not limited to, the participation of regulatory T cells[5,6]. We hypothesized that expression of “protective genes”[5,7] might also promote MS remission. One candidate is heme oxygenase-1 (*HMOX1*/HO-1),

a prototypical cytoprotective and anti-inflammatory stress-responsive gene (reviewed in[5,8]) expressed in the CNS during the course of MS[9] and EAE[10], a well-established model of MS[11].

Under inflammatory conditions, HO-1 becomes the rate-limiting enzyme in the catabolism of heme, yielding equimolar amounts of CO, free Fe, and biliverdin[12], which is subsequently reduced into bilirubin by biliverdin reductase (reviewed in[13]). Induction of HO-1 by metal protoporphyrins has been shown to have salutary effects in a variety of experimental inflammatory conditions (reviewed in[8,14]). The observation that in most cases, exposure to CO can mimic the protective effects of HO-1 (reviewed in[8,15]) would suggest that HO-1 acts in a protective manner via the generation of CO. It is likely, however, that other end products of HO-1 activity such as biliverdin[16] and/or free Fe (by up-regulating heavy chain ferritin expression[17]; or cellular Fe efflux pumps[18]) may exert similar effects.

Whether HO-1 modulates the pathogenesis of autoimmune neuroinflammation remained to be established, because both protective[19] and deleterious[20] effects of chemical HO-1 modulators have been demonstrated in EAE. Using HO-1-deficient (*Hmox1*$^{-/-}$) mice, we demonstrate that expression of HO-1 inhibited inflammation, demyelination, and paralysis, preventing mortality associated with the development of EAE. We also provide evidence that induction of HO-1 using protoporphyrins modulated ongoing autoimmune neuroinflammation, thereby reverting paralysis and leading to disease remission in mice with previously established EAE. Exogenous CO mimicked this effect, which suggests that this end product of heme degradation contributes to the protective action of HO-1. Suppression of EAE was associated with inhibition of (a) leukocyte accumulation in the CNS, (b) MHC class II expression by CNS APC, and (c) pathogenic T_H cell proliferation and effector function.

3. Results

3.1. Expression of HO-1 counters the pathogenesis of EAE

Given that HO-1 expression in the CNS is associated with the development of both EAE and MS[10,19,20], and based on the well-established protective effect of HO-1 (reviewed in[5,8]), we hypothesized that HO-1 might modulate the pathogenesis of EAE. When immunized with the myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}), *Hmox1*$^{-/-}$ C57BL/6 mice developed a more severe form of EAE than did wild-type (*Hmox1*$^{+/+}$) C57BL/6 mice (*Fig.1a* and *Table 1*), with 75% mortality in *Hmox1*$^{-/-}$ mice compared with 19% mortality in *Hmox1*$^{+/+}$ controls (*P* = 0.0024; *Table 1*).

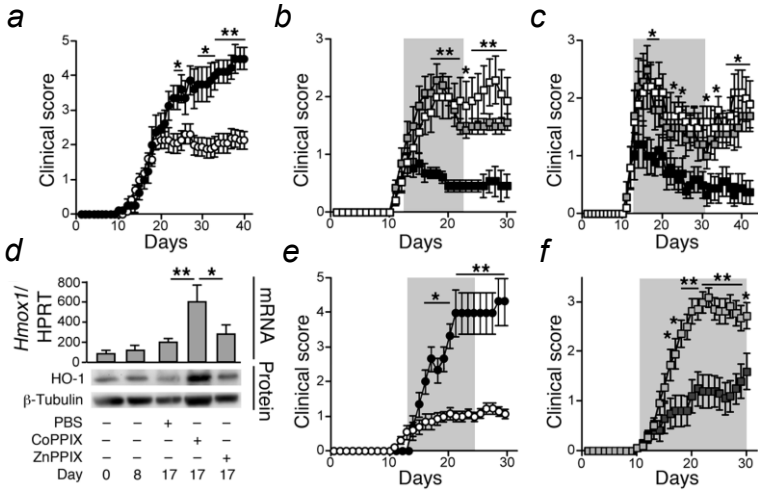

Figure 1. HO-1 and CO suppress EAE progression. EAE was induced, and disease severity was scored daily thereafter. Clinical scores are shown as mean ± SEM. (***a***) Progression of EAE in C57BL/6 *Hmox1*$^{+/+}$ (open circles; *n* = 21) versus *Hmox1*$^{-/-}$ (filled circles; *n* = 8) mice. (***b*** and ***c***) C57BL/6 (***b***) or SJL/J (***c***) mice, randomized 2 days after EAE onset, were treated daily with PBS (open squares; *n* = 10-15), CoPPIX (filled squares; *n* = 10-14), or ZnPPIX (grey squares; *n* = 10-14). (***d***) C57BL/6 *Hmox1*$^{+/+}$ mice were treated as in (b). *Hmox1* mRNA and protein expression in the CNS were assessed by quantitative RT-PCR and Western blotting, respectively. *Hmox1* mRNA expression is shown as mean number of *Hmox1* per HPRT mRNA molecules ± SD. (***e***) EAE was induced in C57BL/6 *Hmox1*$^{+/+}$ (open circles; *n* = 12) and *Hmox1*$^{-/-}$ (filled circles; *n* = 3) mice treated with CoPPIX as in (b). (***f***) EAE was induced in C57BL/6 *Hmox1*$^{+/+}$ mice, randomized 10-12 days after immunization, and exposed to CO (450 ppm; dark grey squares; *n* = 20) or not (light gray squares; *n* = 22). Shaded areas indicate periods of designated treatment. **P* < 0.05; ***P* < 0.01.

Table 1
HO-1 controls the pathogenesis of EAE

Genotype	*n*	Incidence (%)	Onset (d; ± SEM)	Maximal clinical score (± SEM)	Mortal (%)
Hmox1$^{+/+}$	8	95	16 ± 1.01	2.3 ± 0.35 (day 26)	19.0
Hmox1$^{-/-}$	21	100	15.5 ± 0.73	4.5 ± 0.37 (day 40)^{A}	75^{B}

$^{A}P = 0.0053$ versus *Hmox1*$^{+/+}$. $^{B}P = 0.002$ versus *Hmox1*$^{+/+}$.

We tested whether induction of HO-1 by metal protoporphyrins would arrest EAE progression in *Hmox1*$^{+/+}$ C57BL/6 mice. Induction of HO-1 expression and function by administration of cobalt protoporphyrin IX (CoPPIX) after EAE onset (i.e., disease incidence, 37%; mean clinical score, 0.7 ± 0.1) reversed paralysis and led to complete disease remission in 66.6% of mice compared with controls treated with vehicle (PBS) or zinc protoporphyrin IX (ZnPPIX) (*Fig.1b* and *Table 2*). To address whether similar effects would occur in other mouse strains, EAE was induced in SJL/J mice by immunization with the proteolipid protein peptide 139-151 ($PLP_{139-151}$). Induction of HO-1 by administration of CoPPIX after EAE onset in SJL/J mice (disease incidence, 40%; mean clinical score, 0.8 ± 0.2) reversed paralysis and led to complete disease remission in 75% of afflicted mice (*Fig.1c* and *Table 2*). Moreover, disease relapse was also suppressed in SJL/J mice treated with CoPPIX (*Fig.1c* and *Table 2*). That CoPPIX induced HO-1 expression in C57BL/6 mice with established EAE was confirmed at the mRNA and protein levels (*Fig.1d*). CoPPIX induced a 3-fold increase in *Hmox1* mRNA expression in the CNS compared with PBS-treated controls ($P < 0.01$; *Fig.1d*). *Hmox1* mRNA was not significantly induced upon ZnPPIX administration (*Fig.1d*). Expression of HO-1 protein was confirmed using protein extracts from the CNS (*Fig.1d*).

When used at the same dose and schedule shown to arrest EAE progression in *Hmox1*$^{+/+}$ C57BL/6 mice (*Fig.1b*), CoPPIX failed to arrest EAE progression in *Hmox1*$^{-/-}$ C57BL/6 mice (*Fig.1e*), confirming that the protective effect of CoPPIX requires the expression of HO-1.

Table 2
Induction of HO-1 or CO exposure suppresses EAE

Strain	Treatment	*n*	Maximal clinical score (± SEM)	Mortality (%)	Remission (%)
C57BL/6	PBS	15	2.33 ± 0.44 (day 28)	20	0
C57BL/6	CoPPIX	14	0.84 ± 0.21 (day 15)[A]	0	66.6[B]
C57BL/6	ZnPPIX	14	2.28 ± 0.22 (day 18)	20[C]	0
SJL/J	PBS	10	2.3 ± 0.36 (day 20)	0	11.1
SJL/J	CoPPIX	10	1.2 ± 0.45 (day 16)[D]	0	75.0[E]
SJL/J	ZnPPIX	10	2.5 ± 0.44 (day 17)	0	11.1
C57BL/6	Air	10	3.09 ± 0.26 (day 23)	22.7	0
C57BL/6	CO	10	1.9 ± 0.69 (day 30)[F]	15[C]	0

[A]$P = 0.0117$ versus PBS-treated C57BL/6. [B]$P < 0.05$ versus PBS- and ZnPPIX-treated C57BL/6. [C]P = NS. [D]$P = 0.0278$ versus PBS-treated SJL/J. [E]$P < 0.05$ versus PBS- and ZnPPIX-treated SJL/J. [F]$P = 0.009$ versus air-treated C57BL/6.

We have previously shown that exogenous CO can mimic the protective effects of HO-1 in several inflammatory conditions[21] (reviewed in[8]). To determine whether this was the case in EAE, C57BL/6 mice were exposed to CO via inhalation (EAE incidence, 17%; mean clinical score, 0.3 ± 0.1). CO arrested EAE progression and paralysis when compared with air inhaled under similar flow conditions (*Fig.1f* and *Table 2*). We asked whether expression of HO-1 during the course of EAE prevented CNS demyelination, the main cause of paralysis associated with EAE (reviewed in[2-4]). CNS demyelination was more pronounced in *Hmox1*$^{-/-}$ versus *Hmox1*$^{+/+}$ C57BL/6 mice as assessed 60 days after the induction of EAE (*Suppl. Fig.1a-f*). Induction of HO-1 after EAE onset in SJL/J mice prevented CNS demyelination compared with ZnPPIX- or PBS-treated controls (*Fig.2a-c*), an effect consistent with reduction of paralysis (*Fig.1a-c*). Taken together, these observations suggest that the ability of HO-1 to revert paralysis during the progression of EAE is associated with inhibition of CNS demyelination.

3.2. HO-1 modulates the effector function of pathogenic T_H cells

Inhibition of EAE progression and relapse in SJL/J mice treated with CoPPIX was associated with a 87.2% ± 20% (P = 0.0033) and 62.4% ± 40% (P = 0.013) reduction of inflammatory foci in the CNS parenchyma and meninges, respectively,

compared with vehicle‐treated controls (*Fig.2d-g*). ZnPPIX, a protoporphyrin that inhibits HO enzymatic activity, increased by 2.3-fold the total number of inflammatory foci (i.e., parenchyma and meninges) compared with vehicle‐treated controls (P = 0. 0003), suggesting again that endogenous HO‐1 activity counters neuroinflammation associated with EAE progression (*Fig.2d-g*). However, this effect was not reflected by an increase in the clinical scores of the disease (*Fig.1c*).

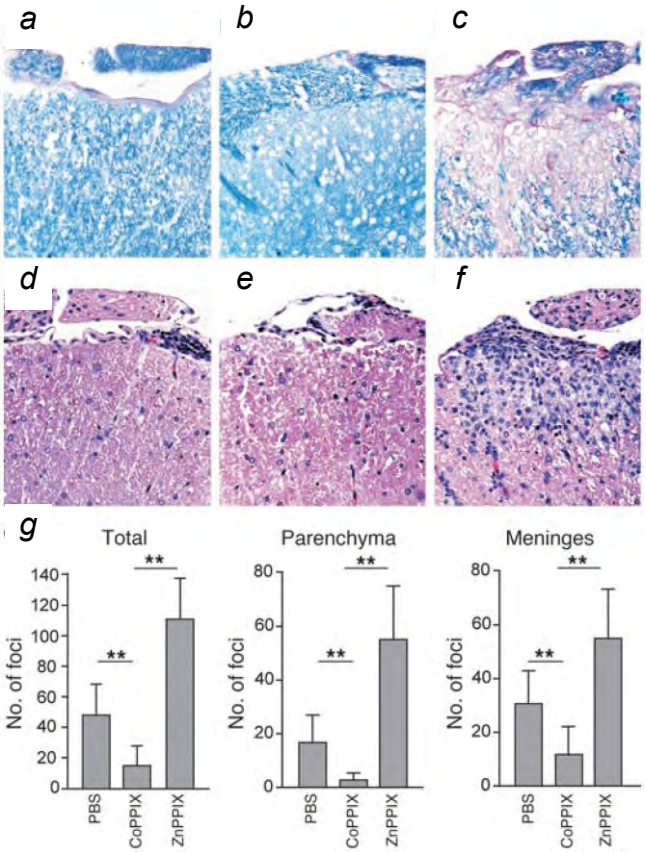

Figure 2. Induction of HO-1 prevents CNS demyelination and formation of inflammatory foci. SJL/J mice, randomized 2 days after EAE onset, were treated daily with PBS (***a*** and ***d***), CoPPIX (***b*** and ***e***), or ZnPPIX (***c*** and ***f***). Representative Luxol fast blue (***a***-***c***) and hematoxylin and eosin (***d***-***f***) spinal cord stainings with adjacent peripheral nerve root are shown 40 days after disease induction. Original magnification, ×160. Note the presence of some edema of myelin (***a***) and meningeal inflammation (***d***) in PBS-treated controls, intact myelin (***b***) and mild meningeal inflammation (***e***) in CoPPIX-treated mice, and demyelination (***c***) and marked meningeal and parenchymal inflammation (***f***) in ZnPPIX-treated mice. (***g***) The number of inflammatory foci in the meninges and parenchyma as well as the total (meninges plus parenchyma) were quantified and are shown as mean ± SD (n = 8-10 per group). $^{**}P < 0.01$.

The ability of HO-1 induction to reduce the formation of inflammatory foci in the CNS (*Fig.2g*) was paralleled by a similar reduction in the total number of leukocytes detected within the CNS. Significant numbers of leukocytes ($CD45^{high}$) were detected in the CNS of C57BL/6 mice with EAE (*Suppl. Fig.2*). These were composed primarily of $CD4^+$ T_H cells, $CD8^+$ T cells, and $CD45^{high}CD11b^+$ macrophages (Mø; *Fig.3a* and *Suppl. Fig.2*). Induction of HO-1 by CoPPIX decreased by 80% ± 6% the total number of leukocytes within the CNS compared with PBS-treated controls ($P < 0.001$; *Fig.3a*). This decrease was reflected by an 83% ± 7% decrease in T_H cells ($P < 0.001$) and a 67% ± 17% decrease in $CD8^+$ T cells ($P < 0.001$) compared with PBS-treated controls (*Fig.3a*). These data demonstrate that HO-1 induction after EAE onset significantly decreases the number of CNS leukocytes and the formation of inflammatory foci within the CNS.

We tested whether HO-1 interfered with the activation, proliferation, and/or acquisition of effector function by CNS-infiltrating T_H cells. Induction of HO-1 by CoPPIX reduced by 57% ± 2% the frequency of T_H cells undergoing cell cycle progression ($BrdU^+$; $P = 0.028$) and by 51.5% ± 34% that of T_H cells expressing IL-2 ($P < 0.01$), compared with vehicle-treated controls (*Fig.3b* and *Suppl. Fig.3a*). This suggests that induction of HO-1 suppresses T_H cell proliferation within the CNS, an effect that should contribute to suppressing EAE progression.

The pathogenic function of CNS-infiltrating T_H cells was also modulated by up-regulation of HO-1 in that HO-1 targeted specifically the expression of IFN-γ in T_H cells. Control-treated mice with EAE had 24% ± 4% CNS-infiltrating T_H cells expressing intracellular TNF-α and IFN-γ (*Suppl. Fig.3b*). Induction of HO-1 by CoPPIX reduced by 60% ± 3% the frequency of T_H cells expressing IFN-γ but failed to affect TNF-α expression in these cells compared with PBS-treated controls (*Fig.3b* and *Suppl. Fig.3b*). The frequency of T_H cells expressing IL-10 (*Fig.3b*) was not modulated by HO-1, an effect that should contribute to suppressing EAE progression as well.

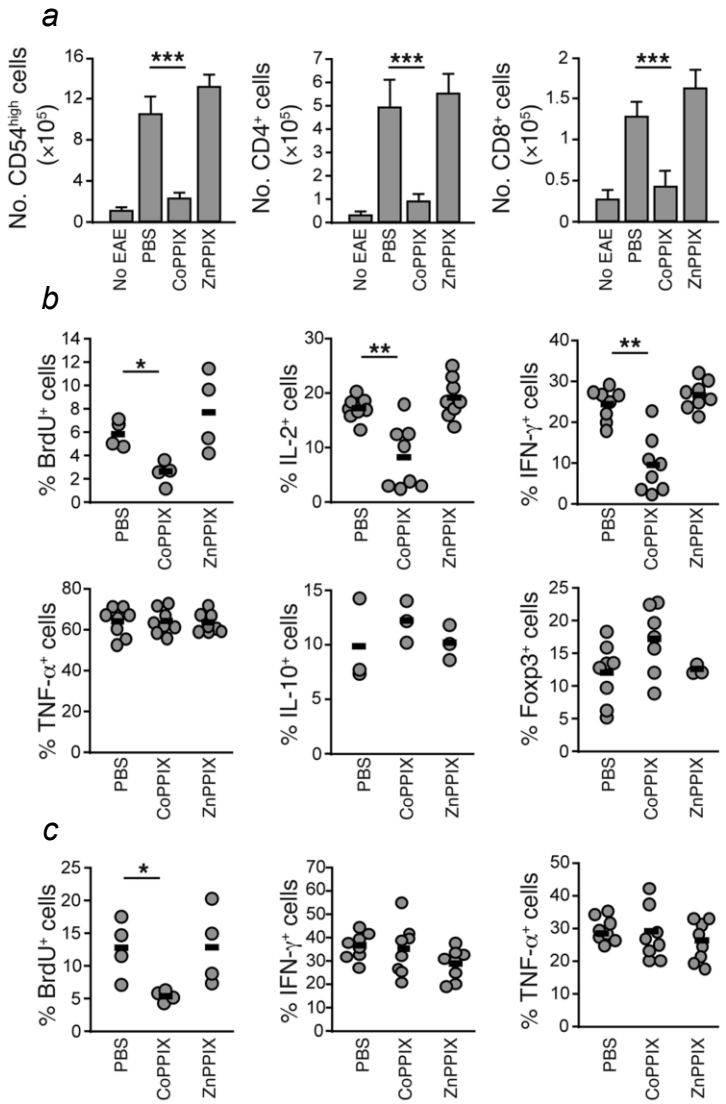

Figure 3. Induction of HO-1 reduces the number of T_H and $CD8^+$ T cells within the CNS and suppresses T_H cell effector function. C57BL/6 mice, randomized 2 days after EAE onset, were treated daily with PBS, CoPPIX, or ZnPPIX. Leukocyte CNS infiltrates were analyzed by flow cytometry 20 days after immunization. (***a***) Number of $CD45^{high}$ (leukocytes), $CD4^+T_H$, and $CD8^+$ T cells are shown as mean ± SD (*n* = 4-5 per group). (***b***) Percentages of CNS $CD4^+$ T_H cells expressing intracellular BrdU (S phase cell cycle progression), IL-2, IFN-γ, TNF-α, IL‑10, and Foxp3 (regulatory T cells). (***c***) Percentage of CNS $CD8^+$ T cells expressing intracellular BrdU, IFN-γ, and TNF-α. Each value in (b) and (c) represents an individual animal. Bars indicate mean values of all mice analyzed in each group. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

We asked whether the protective effect of HO-1 was associated with modulation of regulatory T cells within the CNS. The frequency of forkhead box p3-positive ($Foxp3^+$) regulatory T cells within the CNS was not modulated by HO-1

induction (*Fig.3b* and *Suppl. Fig.3c*), suggesting that the protective effect of HO-1 may act independently of regulatory T cells.

We tested whether expression of T_H cell activation surface markers, including CD69, CD25, CD44, and CD62 ligand (CD62L), were modulated by HO-1 induction. We found that this was not the case for the frequency of T_H cells expressing these markers (*Suppl. Fig.4*) or for their relative level of expression (data not shown).

The frequency of CNS-infiltrating $CD8^+$ T cells undergoing cell cycle progression ($BrdU^+$) was reduced by 55% ± 21% when HO-1 was induced compared with vehicle-treated controls (P = 0.028; *Fig.3c* and *Suppl. Fig.3d*), an effect that should contribute to suppressing EAE progression[2-4]. Induction of HO-1 did not modulate IFN-γ or TNF-α expression by CNS-infiltrating $CD8^+$ T cells (*Fig.3c*).

3.3. HO-1 and CO suppress myelin-reactive T_H cell reactivation

We hypothesized that HO-1 induction might interfere with the reactivation of primed myelin-reactive T_H cells that occurs in the CNS during the development of EAE and presumably also occurs in MS patients. Induction of HO-1 by CoPPIX reduced myelin-reactive T_H cell proliferation by 60.5% ± 3% compared with vehicle- or ZnPPIX-treated controls (P < 0.001; *Fig.4a*). Moreover, HO-1 induction did not affect the proliferative response of draining lymph node T_H cells to concanavalin A (ConA; *Suppl. Fig.5a*) nor did it reduce T_H cell numbers in draining lymph nodes (data not shown).

In the same experimental system, induction of HO-1 suppressed secretion of TNF-α by 64% ± 2% (P < 0.001), IFN-γ by 75% ± 2% (P < 0.001), and IL-12/23(p40) by 61% ± 16% (P < 0.01) compared with controls (*Fig.4b*). This suggests that HO-1 suppresses the differentiation of primed myelin-reactive T_H cells toward a pathogenic effector phenotype characterized by the secretion of pro-inflammatory cytokines.

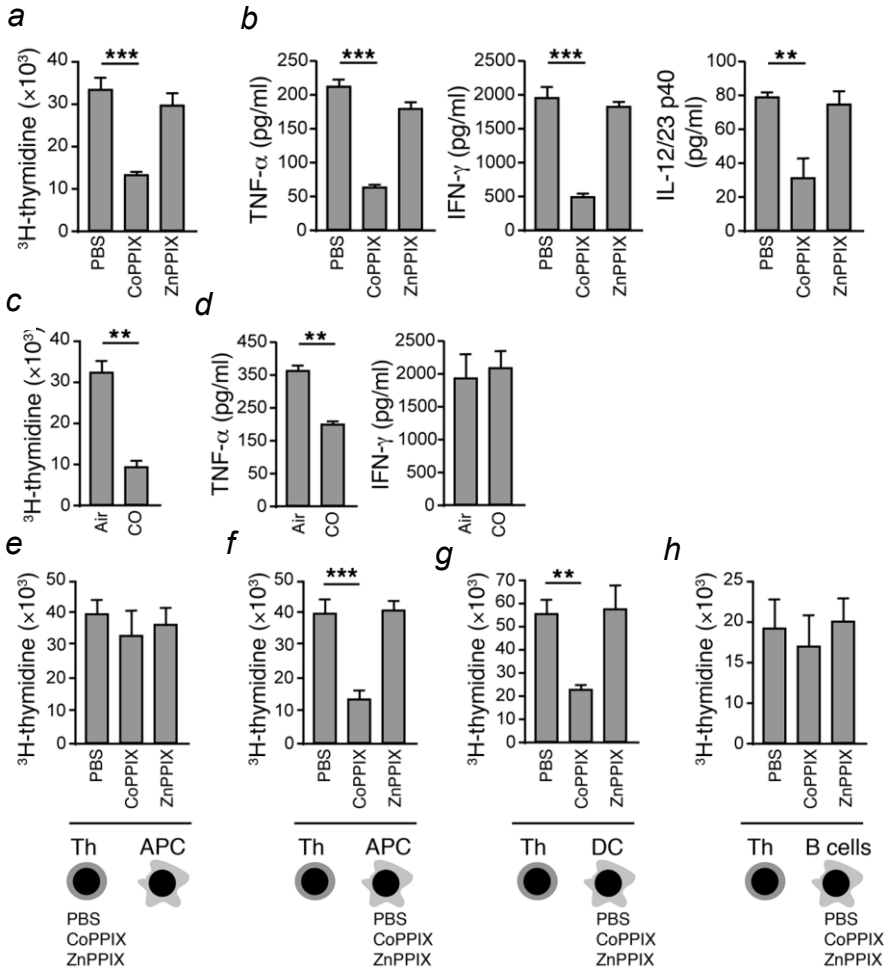

Figure 4. The antiproliferative effect of HO-1 is exerted via APC. Results shown are mean ± SD from triplicate samples in 1 of at least 3 independent assays, except for IL-12/23(p40) (1 assay). (***a***) C57BL/6 mice were treated as described in Methods. Eight days after immunization, proliferation of myelin-reactive T_H cells was assessed *in vitro* 72 hours after addition of MOG_{35-55} (10 μg/ml). (***b***) Cytokines were assayed in the cell culture supernatants (100 μg/ml MOG_{35-55} for 72 hours). (***c***) C57BL/6 mice were exposed to air (n = 3) or CO (450 ppm; n = 3) starting 2 days prior to immunization. T_H cell proliferation was assayed as in (a). (***d***) Mice were treated as in (c), and cytokines were assayed as in (b). (***e-h***) C57BL/6 mice were treated as in (a). (***e***) T_H cells (>98% CD4$^+$) from PBS-, CoPPIX-, or ZnPPIX-treated mice (n = 5) were cocultured with APC (<98% CD4$^+$) from immunized but otherwise untreated mice. T_H cell proliferation was measured as in (*a*). T_H cells from untreated immunized mice were cocultured with (***f***) APC (<2% CD4$^+$), (***g***) DC (>98% CD11c$^+$), or (***h***) B cells (>98% B220$^+$) from PBS-, CoPPIX-, or ZnPPIX-treated immunized mice (n = 5). T_H cell proliferation was measured as in (a). $^{**}P < 0.01$; $^{***}P < 0.001$.

To assess whether suppression of T_H cell reactivation *in vitro* was due to defective priming of naïve myelin-reactive T_H cells *in vivo*, draining lymph node T_H cells (>98% CD4$^+$) were isolated 7 days after immunization and rechallenged *in vitro* with MOG_{35-55} in the presence of naïve APC (<2% CD4$^+$). Proliferation of

myelin-reactive T_H cells from CoPPIX-treated mice was similar to that of control mice (*Suppl. Fig.5a*), suggesting that HO-1 induction did not interfere with priming of naïve myelin-reactive T_H cells *in vivo*.

Exposure of C57BL/6 mice to CO inhibited by 71% ± 5% the proliferative response of myelin-reactive T_H cells compared with control mice exposed to air under similar flow conditions ($P = 0.0016$; *Fig.4c*). In the same experimental setting, CO inhibited TNF-α secretion by 40% ± 2% ($P = 0.0029$) but failed to inhibit IFN-γ secretion (*Fig.4d*). This suggests that the immunomodulatory effect of CO may be more restricted than that of HO-1 induction.

3.4. HO-1 expression in APC inhibits myelin-reactive T_H cell proliferation

We reasoned that inhibition of T_H cell proliferation could result from induction of HO-1 expression in T_H cells[22,23] and/or in APC[24]. To test the first possibility, T_H cells were purified from MOG_{35-55}-immunized mice in which HO-1 was induced. Proliferation of T_H cells from CoPPIX-treated mice was not significantly inhibited in the presence of APC isolated from immunized but otherwise untreated mice compared with PBS- or ZnPPIX-treated controls (*Fig.4e*). This suggests that HO-1 expression in T_H cells is not sufficient *per se* to suppress T_H cell proliferation.

That the anti-proliferative effect of HO-1 is exerted via APC is suggested by the following set of observations. Proliferation of purified myelin-reactive T_H cells from immunized but otherwise untreated mice was inhibited by 65% ± 7% in the presence of purified APC from immunized mice treated with CoPPIX compared with control APC isolated from PBS- or ZnPPIX-treated immunized controls ($P < 0.001$; *Fig.4f*). T_H cell proliferation was inhibited by 59% ± 3% ($P < 0.01$) by DC (>98% $CD11c^+$; *Fig.4g*) but not by B cells (>98% $CD19^+$; *Fig.4h*) isolated from CoPPIX-treated immunized mice compared with DC or B cells isolated from control PBS- or ZnPPIX-treated immunized mice, respectively.

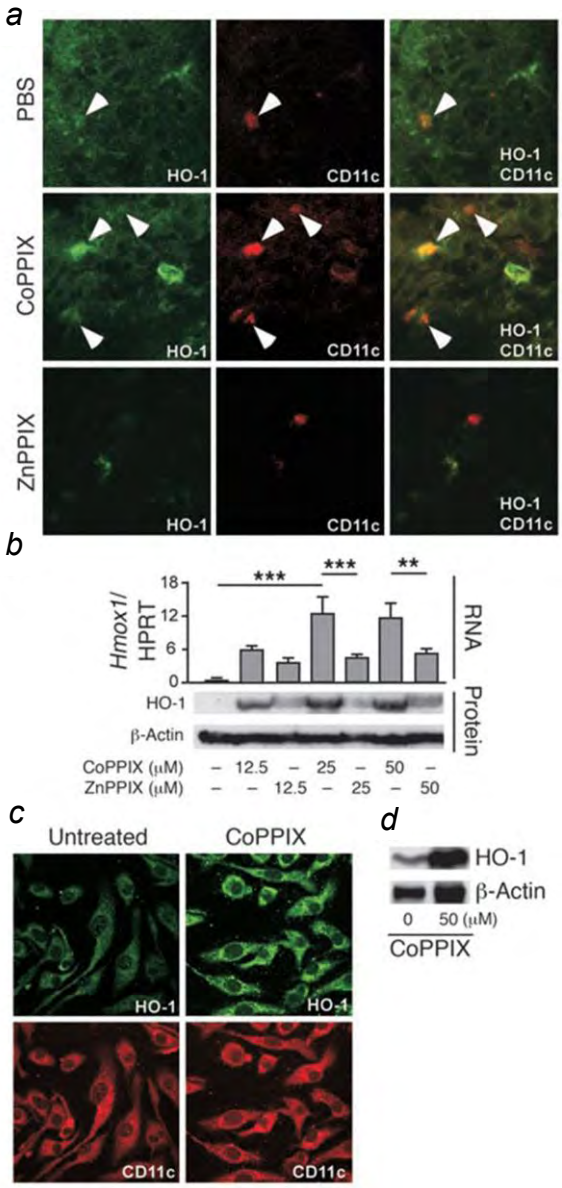

Figure 5. CoPPIX induces HO-1 expression in DC *in vivo* and *in vitro*. (***a***) C57BL/6 mice were randomized 2 days after EAE onset and treated daily with PBS, CoPPIX, or ZnPPIX. HO-1 and CD11c (DC) expression in spinal cords was detected by immunocytochemistry 7 days after beginning treatments and analysed by confocal microscopy. Shown are HO-1 (left panels), CD11c (middle panels) and CD11c plus HO-1 (right panels). Original magnification, ×240. Arrows indicate positive staining. (***b***) Unsorted bone marrow-derived DC (approximately 80% $CD11c^+$) were exposed to CoPPIX or ZnPPIX, and *Hmox1* mRNA and protein expression were assessed by quantitative RT-PCR and Western blotting, respectively. *Hmox1* mRNA is shown as mean number of *Hmox1* per HPRT mRNA molecules ± SD (n = 3 per group). (***c***) Bone marrow-derived DC were purified (>98% $CD11c^+$) and exposed to CoPPIX (50 μM for 16 hours) as in (b). HO-1 (green) and CD11c (red) were detected as in (a). Original magnification, ×400. (***d***) Expression of HO-1 was detected by Western blot in purified DC shown in (c). $^{**}P < 0.01$; $^{***}P < 0.001$.

Administration of CoPPIX to C57BL/6 mice after EAE onset induced high levels of HO-1 expression in CNS-infiltrating DC ($CD11c^+$) compared with PBS- or ZnPPIX-treated controls (*Fig.5a*). Similar effects were observed *in vitro*: CoPPIX induced a 64-fold increase in *Hmox1* mRNA expression in bone marrow-derived DC (approximately 80% $CD11c^+$) compared with vehicle-treated controls (*Fig.5b*). While *Hmox1* mRNA expression was also induced by ZnPPIX, this effect was significantly less pronounced than that of CoPPIX (*Fig.5b*). These observations are consistent with the notion that ZnPPIX can induce HO-1 expression moderately while inhibiting its activity strongly, thus acting as a potent HO-1 inhibitor[25]. In the same experimental setting, CoPPIX induced HO-1 protein expression while ZnPPIX failed to do so (*Fig.5b*). To ensure that CoPPIX induced HO-1 expression specifically in DC and not in a putative contaminating cell population, bone marrow-derived DC were further purified (>98% $CD11c^+$). That CoPPIX induced HO-1 expression specifically in $CD11c^+$ cells was shown by immunocytochemistry (*Fig.5c*) and Western blot (*Fig.5d*). These data demonstrate that CoPPIX, but not ZnPPIX, induces HO-1 expression in DC, a finding concordant with those of others[24].

3.5. HO-1 suppresses MHC class II expression in activated APC

To evaluate further the impact of HO-1 in APC function, draining lymph node DC from immunized C57BL/6 mice were analyzed by flow cytometry. Induction of HO-1 by CoPPIX reduced by 39% ± 12% MHC class II expression in DC ($CD11c^+$) compared with controls ($P < 0.001$; *Fig.6a*). This effect was specific to MHC class II, as expression of CD40, CD80, and CD86 were not significantly affected (*Suppl. Fig.6a*). Induction of HO-1 did not alter the frequency or total number of DC in the draining lymph nodes of immunized mice (data not shown). Exogenous CO inhibited by 24% ± 8% MHC class II expression in DC compared with air-treated controls ($P = 0.0011$; *Fig.6b*). This effect was again specific to MHC class II, as CO failed to inhibit the expression of CD40, CD80, or CD86 (*Suppl. Fig.6b*).

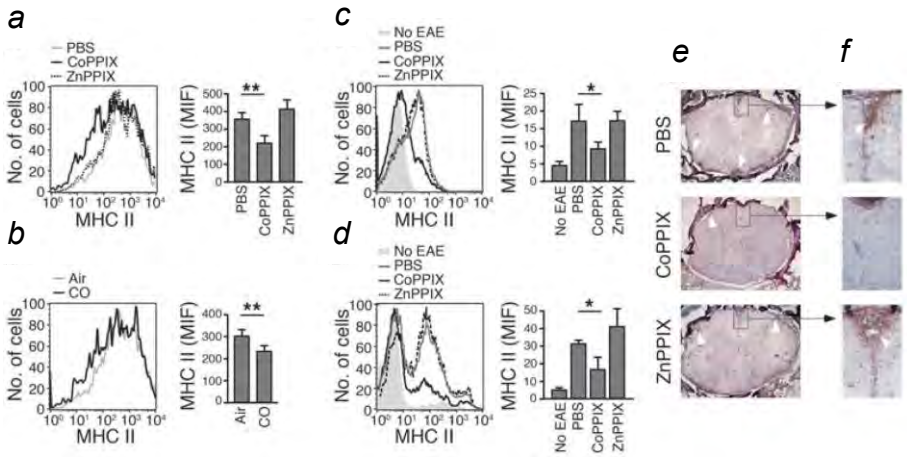

Figure 6. Induction of HO-1 and exposure to CO inhibit MHC class II expression in APC. (***a***) C57BL/6 mice were treated daily with PBS, CoPPIX, or ZnPPIX (*n* = 4-6) starting 2 days prior to footpad immunization. Draining lymph node cells were isolated, and surface MHC class II expression was analyzed in DC ($CD11c^+$) by flow cytometry 8 days after immunization. Representative histograms and quantifications (mean intensity of fluorescence; MIF) are shown as mean ± SD. (***b***) C57BL/6 mice were exposed to air (*n* = 6) or CO (450 ppm; *n* = 7) starting 2 days prior to immunization and continuously thereafter. Draining lymph node cells were isolated, and surface MHC class II expression was assessed in DC ($CD11c^+$) as in (a). Representative histograms and quantification are shown as mean ± SD. (***c*** and ***d***) C57BL/6 mice, randomized 2 days after EAE onset, were treated daily with PBS, CoPPIX, or ZnPPIX (*n* = 9 per group). MHC class II expression in (***c***) microglia ($CD45^{low}CD11b^+$) and (***d***) CNS-infiltrating Mø ($CD45^{high}CD11b^+$) was analyzed by flow cytometry 20 days after immunization, when controls, i.e., ZnPPIX and PBS, reached maximal disease severity. Representative histograms and quantifications are shown as mean ± SD. (***e*** and ***f***) EAE induction and treatments were performed as in (c and d). MHC class II expression was detected by immunocytochemistry and counterstained. Original magnification, ×10 (***e***); ×40 (***f***). White arrows indicate MHC class II expression. **P* < 0.05; ***P* < 0.01.

To test whether HO-1 and/or CO would affect other APC populations involved in the pathogenesis of EAE[26,27], MHC class II expression was analyzed in microglia ($CD45^{low}CD11b^+$) and CNS-infiltrating Mø ($CD45^{high}CD11b^+$) of C57BL/6 mice with established EAE. Induction of HO-1 starting 2 days after disease onset (approximately 40% EAE incidence) reduced MHC class II expression by 44% ± 13% in microglia (*P* < 0.05; *Fig.6c*) and by 47% ± 24% in CNS-infiltrating Mø (*P* < 0.05; *Fig.6d*) compared with controls.

MHC class II-expressing cells in the CNS of mice with established EAE was confined to the perivascular area and associated primarily with CNS-infiltrating leukocytes, i.e., Mø (*Fig.6e,f*). Low level MHC class II expression was also detected in the parenchyma, presumably associated with microglia (data not shown). Induction of HO-1 by CoPPIX reduced MHC class II expression in both perivascular

leukocytes and microglia (*Fig.6e,f*), confirming similar observations made by flow cytometry.

To address further the mechanism by which HO-1 suppressed MHC class II expression in CNS APC, we tested whether induction of HO-1 in microglial BV2 cells would inhibit MHC class II expression. When stimulated *in vitro* with IFN-γ, microglial BV2 cells up-regulated the expression of MHC class II (*Fig.7a,b*). Induction of HO-1 by CoPPIX inhibited by 27% ± 7% MHC class II expression compared with controls ($P < 0.05$; *Fig.7a*). Exposure to CO mimicked this effect, inhibiting by 20% ± 7% MHC class II expression compared with air-treated controls ($P < 0.05$; *Fig.7b*). Induction of HO-1 or exposure to CO had no effect on CD40, CD80, or CD86 expression in BV2 cells (data not shown). Induction of HO-1 inhibited IFN-γ-driven phosphorylation of STAT-1 without affecting STAT-1 expression (*Fig.7c*). Induction of MHC class II transcription activator (CIITA) mRNA expression by IFN-γ was inhibited by 30% ± 11% compared with controls ($P < 0.05$; *Fig.7d*). Given the central role of STAT-1 phosphorylation and CIITA expression in the transcriptional regulation MHC class II expression in APC[28] (reviewed in[29]), the inhibitory effect of HO-1 over MHC class II expression in these cells might be explained by its ability to suppress STAT-1 phosphorylation and CIITA expression.

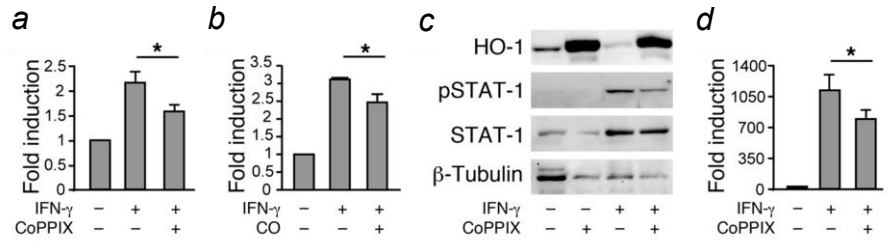

Figure 7. HO-1 inhibits STAT-1 phosphorylation and CIITA expression in CNS APC. (***a***) and (***b***) Expression of MHC class II in microglial BV2 cells was monitored by flow cytometry. When indicated (+), BV2 cells were exposed to CoPPIX (50 μM) or CO (250 ppm) 6 or 16 hours, respectively, before IFN-γ stimulation (50 U/ml for 24 hours). MHC class II expression is shown as fold induction *versus* untreated cells ± SD ($n = 3 - 7$). (**c**) Phosphorylated STAT-1, total STAT-1, HO-1, and β-tubulin were detected by Western blot in BV2 cells treated as in (a). (***d***) Expression of *CIITA* mRNA was quantified by real-time PCR in BV2 cells treated as in (a). Results are shown as mean fold induction *versus* untreated cells ± SD ($n = 3$). *$P < 0.05$.

4. Discussion

There are several genes that can limit the deleterious effects of inflammation and thus counter the pathogenesis of inflammatory diseases[5,7]. We refer to those genes as protective[7] and hypothesized that the protective gene HO-1 may control the pathologic outcome of EAE, a well-established model of MS. We provide evidence that endogenous HO-1 suppressed both the severity and the mortality associated with the development of this neuroinflammatory disease (*Fig.1a* and *Table 1*). Upregulation of HO-1 by CoPPIX acted therapeutically to reverse paralysis (*Fig.1b,c* and *Table 2*). That the therapeutic effect of CoPPIX is exerted via HO-1 is demonstrated by the observation that (a) CoPPIX induces the expression of HO-1 in the CNS of mice with EAE (*Fig.1d*) and (b) the protective effect of CoPPIX is abrogated in *Hmox1*$^{-/-}$ mice (*Fig.1e*).

CO contributes to the protective effects of HO-1, as exposure of *Hmox1*$^{+/+}$ mice to CO mimicked the protective effects of HO-1 (*Fig.1f*, *Fig.4c,d*, *Fig.6b*, and *Fig.7b*). One would expect, however, that other end products of heme degradation, e.g., biliverdin, may act in a similar manner[30]. However, biliverdin does not suppress myelin-reactive T_H cell proliferation, nor does it suppress EAE progression (*Suppl. Fig.7*). One possible explanation may be that the protective effect of HO-1 is exerted within the CNS, which biliverdin cannot enter because of its exclusion by the blood-brain barrier.

Our data suggest that the mechanism underlying the protective effect of HO-1 is associated with a profound inhibition of leukocyte accumulation (*Fig.2g* and *Fig.3a*) and reactivation (*Fig.3b,c*) within the CNS. This is suggested by the observation that HO-1 induction by CoPPIX administration after EAE onset reduced the number of inflammatory foci (*Fig.2g*) as well as the total number of CNS T_H cells, $CD8^+$ T cells, and Mø (*Fig.3a*) and in addition suppressed the proliferation of the remaining T_H and $CD8^+$ T cells (*Fig.3b,c*) infiltrating the CNS.

The anti-proliferative effect of HO-1 was shown *in vitro* to be strictly dependent on DC (*Fig.4g*) that express high levels of HO-1 (*Fig.5b,c*). That a

similar effect may occur *in vivo* to arrest EAE progression is suggested by the observation that DC expressing high levels of HO-1 were detected in the CNS of CoPPIX-treated mice in which EAE progression was arrested (*Fig.5a*).

Induction of HO-1 leads to specific inhibition of MHC class II in APC, including DC (*Fig.6a,b*), microglia (*Fig.6c*), and CNS-infiltrating Mø (*Fig.6d*). Induction of HO-1 expression in microglia suppressed STAT-1 phosphorylation as well as CIITA expression, two critical events for MHC class II expression in APC[28] (reviewed in[29]) as well as in the reactivation of myelin-reactive T_H cells in the CNS[31]. This effect is likely to contribute to the overall protective effect of HO-1 induction, as MHC class II expression in microglia is thought to be involved in EAE pathogenesis and progression (reviewed in[32,33]).

That inhibition of MHC class II accounts for the protective effect of HO-1 is supported by the association between certain human MHC class II locus alleles and MS susceptibility (reviewed in[34]). Moreover, peptides that interfere directly with MHC class II-mediated antigen presentation to myelin-reactive T_H cells are very efficient in arresting EAE progression[35] (reviewed in[2]), suggesting again that inhibition of MHC class II expression by APC is probably sufficient to explain the protective effects of HO-1 observed herein. Another possibility would be that HO-1 promotes the accumulation of regulatory T cells and/or their activity within the CNS. This would be consistent with widespread evidence that regulatory T cells can control the pathogenesis of EAE[6,36-38] as well as with the hypothesis that HO-1 expression may control regulatory T cell function[39]. However, we observed that despite its ability to suppress ongoing EAE, induction of HO-1 failed to modulate the number of CNS-infiltrating regulatory T cells (*Fig.3b*). Furthermore, we also found that the number and function of regulatory T cells was unaffected in naïve *Hmox1*$^{-/-}$ mice compared with *Hmox1*$^{+/+}$ controls, suggesting that HO-1 does not influence regulatory T cell development and/or function[40]. Taken together, these data suggest that the protective effect of HO-1 in EAE does not act via modulation of regulatory T cells.

That HO-1 exerts its protective effects via APC, including CNS microglia (*Fig.6c,d*), is relevant for its mechanism of action if one considers that

immunomodulation within the CNS is probably required to arrest MS progression. This may explain the relative lack of efficiency in treating MS by controlling exclusively peripheral antigen presentation, which is probably not as relevant, whereas prevention of effector T_H cell reactivation by CNS APC almost certainly is[26,31]. Our finding that induction of HO-1 after EAE onset suppressed T_H cell reactivation and effector function within the CNS (*Fig.4b*) may explain why this approach was effective in suppressing disease progression (*Fig.1b,c* and *Table 2*).

Our present data suggest that upon induction of HO-1 in APC, the effector function of myelin-reactive T_H cells in the CNS is modulated in a manner that suppresses their pathogenicity. This is supported by the suppression of neuroinflammatory, i.e., IFN-γ, but not neuroprotective, i.e., IL-10[41] and TNF-α[42], cytokine expression by CNS-infiltrating T_H cells (*Fig.3b*). Presumably, inhibition of high-level IFN-γ expression by CNS-infiltrating T_H cells should promote oligodendrogenesis and thus aid to EAE remission[43]. It should be noted, however, that while inhibition of IFN-γ expression in CNS-infiltrating T_H cells is likely to contribute to EAE regression[44], this remains to be formally established as EAE is exacerbated in IFN-γ-deficient mice[45].

One possibility not excluded by the present study is that HO-1 may prevent EAE progression not only by immunomodulation but also by its cytoprotective properties[46,47] in the CNS, i.e., oligodendrocytes or neurons[48]. Such an effect would be consistent with the observed arrest of EAE progression[49] as well as with our previous observation that cytoprotection afforded by HO-1 can prevent the rejection of transplanted organs[50].

Even with the caution necessary for extrapolating from EAE to MS, there are several independent lines of evidence suggesting that HO-1 expression affects the clinical outcome of MS. First, HO-1 is expressed in the CNS of MS patients[9]. Second, HO-1 prevents the deleterious effects of inflammation in humans[51]. Third, a (GT)n microsatellite polymorphism in the human *HMOX1* promoter controls HO-1 inducibility and dictates the incidence of several inflammatory diseases (reviewed

in[52]). Additional studies are needed to determine whether HO‑1 functions to prevent MS progression or promote its remission.

In conclusion, we found that HO-1 suppresses the pathologic outcome of autoimmune neuroinflammation associated with the development of EAE. This effect is mediated at least in part by CO, which acts on APC to inhibit the expression of MHC class II and presumably the reactivation of pathogenic T_H cells within the CNS. We suggest that modulation of HO-1 expression or administration of CO may be a useful therapeutic strategy to treat MS patients.

5. Methods

Animals. C57BL/6 and SJL/J mice were maintained under specific pathogen-free conditions approved by the Animal User and Institutional Ethical Comities of the Instituto Gulbenkian de Ciência and the Beckman Center for Molecular Medicine. Mice were used between 6 and 8 weeks of age. *Hmox1*$^{+/-}$ mice were originally generated by S.-F. Yet (Brigham and Women's Hospital, Boston, Massachusetts, USA[53]). Littermate *Hmox1*$^{+/-}$ and *Hmox1*$^{+/+}$ mice were used as controls.

Cells and reagents. Microglial BV2 cells, obtained from E. Blasi (University of Modena and Reggio Emilia, Modena, Italy), were cultured essentially as described previously[54]. Recombinant mouse IFN-γ (PeproTech) was used to induce MHC class II expression in BV2 cells. MOG_{35-55} was synthesized at the Biopolymers Laboratory of Harvard Medical School. $PLP_{139-151}$ was synthesized at the PAN Facility of the Beckman Center for Molecular and Cellular Medicine. CoPPIX, ZnPPIX (Frontier Scientific Inc.), and biliverdin hydrochloride (MP Biomedicals) were dissolved in 0.2 N NaOH, neutralized with 0.2 N HCl, adjusted to 1 mg/ml (CoPPIX and ZnPPIX) and 10 mM (biliverdin) with distilled water, and sterilized by filtration.

Cytokine assays. Cell culture supernatants were used to measure TNF-α, IFN-γ, and IL-12/23(p40) concentrations by ELISA according to the manufacturer's indications (OptEIATM; BD Biosciences - Pharmingen).

CNS leukocyte infiltration. Leukocytes were isolated from the CNS as described previously[55]. The total number of $CD45^{high}$, $CD11b^{+}$, $CD4^{+}$, and $CD8^{+}$ T cells in the CNS was assessed by flow cytometry, using a fixed number of latex beads (Beckman Coulter) coacquired with a pre-established volume of the cellular suspensions.

DC. Bone marrow from naïve mice was flushed, and single-cell suspensions were cultured (37ºC; 5% CO2, 95% humidity) in RPMI 1640 (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen), and 1% GM-CSF conditioned medium. Medium was replaced every 48 hours until day 6 of culture, after which CD11c cells were purified as described above or remained untreated.

EAE induction and protoporphyrin treatment. Briefly, C57BL/6 and SJL/J mice were immunized subcutaneously with MOG_{35-55} or $PLP_{139-151}$ (200 μg), respectively, emulsified in CFA (BD Diagnostics) supplemented with Mycobacterium tuberculosis (400 μg; BD Diagnostics). C57BL/6 mice received Pertussis toxin (200 ng i.v.; Sigma-Aldrich) at the time of immunization and 2 days thereafter. Clinical signs of EAE were evaluated daily and scored as follows: 0, normal; 1, limp tail; 2, partial paralysis of the hind limbs; 3, complete paralysis of the hind limbs; 4, hind-limb paralysis and forelimb weakness; 5, moribund or deceased. Protoporphyrins were administered daily (200 μl i.p., 5 mg/kg). Biliverdin was administered at 5 μM/kg every 12 hours.

Flow cytometry and antibodies. Surface markers and intracellular cytokines were detected essentially as described previously[55]. Purified anti-mouse CD4 (RM4-5), CD8 (YTS169.4), CD11b (M1/70), CD11c (HL3), CD40 (3/23), CD45 (30-F11), CD80 (16-10A1), CD86 (GL1), B220 (RA3-6B2), I-Ab (AF6-120.1), IL-2 (JES6-5H4), IL-10 (JES5-16E3), TNF-α (MP6-XT22), and IFN-γ (XMG1.2; all BD Biosciences - Pharmingen) were used. Anti-FcγIII/II receptor mAbs were prepared in house from hybridoma (2.4G2) culture supernatants. Antibodies were directly conjugated to PE, allophycocyanin, or FITC.

***In vivo* BrdU incorporation.** Mice received BrdU (50 μg/g body weight i.p. administered 4 times every 2 hours; BD Biosciences - Pharmingen). CNS leukocyte infiltrates were isolated as described above, and nuclear BrdU was detected using the FITC-labeled anti-BrdU Flow Kit according to the manufacturer's indications (BD Biosciences - Pharmingen).

Leukocyte isolation and purification. Draining lymph nodes were homogenized into single-cell suspension. $CD4^+$ and $CD11c^+$ cells were purified using single-step anti-CD4 (L3T4) and anti-CD11c (N418) MicroBeads, respectively (Miltenyi Biotec). $CD8^+$ and $CD19^+$ cells were purified by two-step labeling consisting of FITC-labeled anti-CD8 (SK1) and anti-CD19 (4G7) mAb (BD) followed by anti-FITC-conjugated MicroBeads (Miltenyi Biotec). Cells were separated using a MidiMACS magnetic isolation system (Miltenyi Biotec), and purity was assessed by flow cytometry.

MOG_{35-55}-reactive T_H cell proliferation. Draining lymph node cells were isolated from PBS-, CoPPIX-, or ZnPPIX-treated animals 8 days after footpad immunization (MOG_{35-55} plus CFA). Cells were plated in 96-well microtiter plates (5×10^5 cells per well) in RPMI 1640 (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS, 50 μM 2-mercaptoethanol (2-ME), 10 mM HEPES, and 1 mM sodium pyruvate (all from Invitrogen) and exposed to MOG_{35-55} (10 μg/ml) or concanavalin A (2 μg/ml; Sigma-Aldrich) for 72 hours at 37ºC (5% CO2, 95% humidity). Cell proliferation was assessed by [^{3}H]-thymidine (1 μCi/well; GE HealthCare) incorporation during the last 6 hours of culture evaluated in a scintillation counter (Tomtec; Pharmacia).

Immunocytochemistry. Purified bone marrow-derived DC were plated in glass coverslips (Paul Marienfeld GmbH & Co.) and fixed in acetone (10 minutes at -20ºC). Rabbit anti-HO-1 polyclonal antibody (SPA895; Stressgene Biotechnologies), PE-labeled anti-CD11c mAb (HL3; BD Biosciences - Pharmingen) and FITC-labeled goat anti-rabbit polyclonal antibody (Sigma-Aldrich) were used. Slides were mounted in Vectashield (Vector Laboratories), and fluorescence was

detected by confocal microscopy (Leica Spectral TCS-SP2; Leica Microsystems). Images were acquired using Leica confocal software (version 2.61).

Histology. Mice were perfused with PBS followed by 10% formalin. Brain and spinal cord sections were embedded in paraffin and stained with hematoxylin and eosin or with Luxol fast blue stains, and inflammatory foci were enumerated in meninges and parenchyma as described previously[56].

Immunohistology. Mice were perfused with PBS, and their spinal cords were harvested, embedded in Tissue-Tek OCT (Sakura), and snap-frozen in liquid nitrogen. Spinal cord sections (10 μm) were fixed in acetone, and antigen detection was performed essentially as described previously. Rabbit anti-HO-1 polyclonal antibody (SPA895; Stressgene Biotechnologies), biotin-labeled anti-MHC class II mAb (AF6-120.1; BD Biosciences - Pharmingen), PE-labeled anti-CD11c mAb (HL3; BD Biosciences - Pharmingen), and FITC‑labeled goat anti‑rabbit (Sigma-Aldrich) polyclonal antibody were used. HRP-conjugated streptavidin and Vectastain Elite ABC kit were used according to the manufacturer's instructions (Vector Laboratories). HRP stainings were revealed using 3,3-diamenobenzidine (Sigma-Aldrich), and tissues were counterstained with Harris hematoxylin (Sigma-Aldrich). Images were acquired using a Leica (DM.LB2) microscope (Leica Microsystems) equipped with an Evolution MP5.0 color camera (MediaCybernetics) and free‑GIMP 2.2.10 software (http://portableapps.com/apps/graphics_pictures /gimp_portable). Fluorescence staining was detected and processed as described above.

CO exposure. Mice were placed in a plexiglass gastight 60-l capacity chamber and exposed continuously to CO as described previously[47]. Cells (37ºC, 95% humidity) were exposed to CO (250 ppm in air, 5% CO_2) in a plexiglass gastight 10-l chamber (2 l/min). CO concentration was monitored using a CO analyzer (Interscan Corporation). Air controls were maintained in a similar chamber without CO.

Western blots and antibodies. Western blots were performed as described previously[46,47] using anti-HO-1 (SPA896; Stressgene Biotechnologies), anti-STAT-1 (Upstate USA Inc.), and anti-phospho-STAT-1 (Upstate USA Inc.) rabbit polyclonal antibodies as well as anti-β-tubulin (Sigma-Aldrich) and anti-β-actin (Sigma-Aldrich) mouse mAbs. HRP-labeled goat anti-rabbit (31460; Pierce Biotechnology) or goat anti-mouse (31439; Pierce Biotechnology) polyclonal antibodies were used to detect the primary antibodies. HRP activity was revealed using ECL. Images were acquired using a Kodak 440CF image station.

Real-time PCR. Total RNA was extracted using RNeasy Protect MiniKit (Qiagen) according to the manufacturer's instructions and reverse transcribed as described previously[22]. HO-1 (5′-TCTCAGGGGGTCAGGTC-3′ and 5′-GGAGCGGTGTCTGGGATG-3′), CIITA (5′-CTCTACCACCTCTATGACC-3′ and 5′-GCTTCTGTCCTGCTTCTAC-3′), and hypoxanthine-guanine phosphoribosyl transferase (HPRT; 5′-GTTGGATACAGGCCA GACTTTGTTG-3′ and 5′-GATTCAACCTTGCGCTCATCTTAGGC-3′) PCR products were detected using LightCycler realtime quantitative PCR (Roche Diagnostics) as described previously[22].

Statistics. Significance of clinical scores was examined by the Mann-Whitney test. Significance of *Hmox1*$^{-/-}$ versus *Hmox1*$^{+/+}$ mouse survival was examined by the log-rank test. Fisher's exact test was used for analyses of disease remission. All other statistical analyses were performed using ANOVA with Bonferroni's correction for multiple comparisons. $P < 0.05$ was considered significant in all tests.

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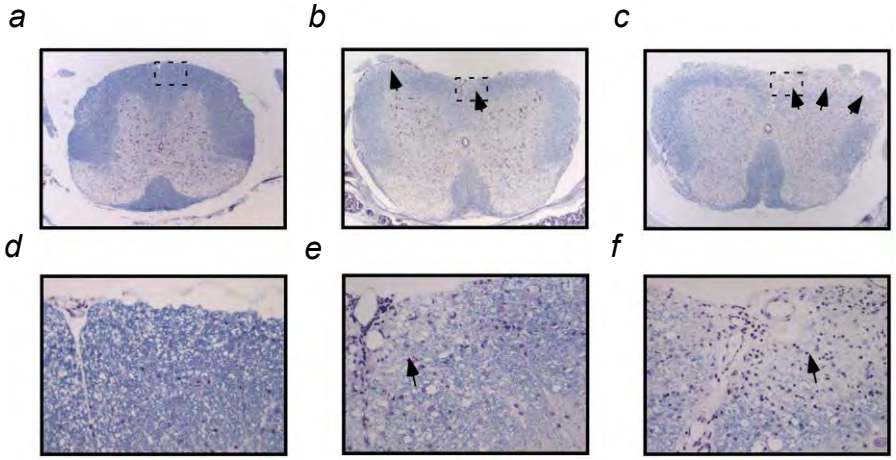
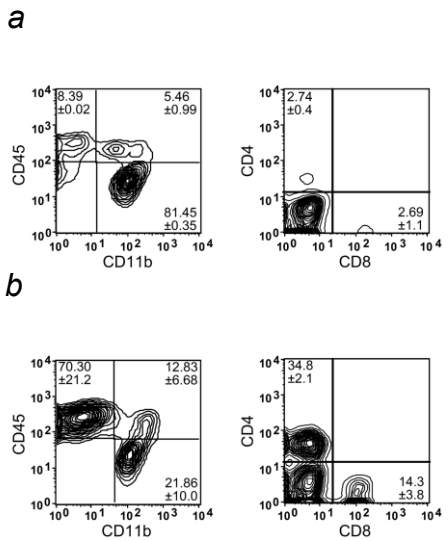
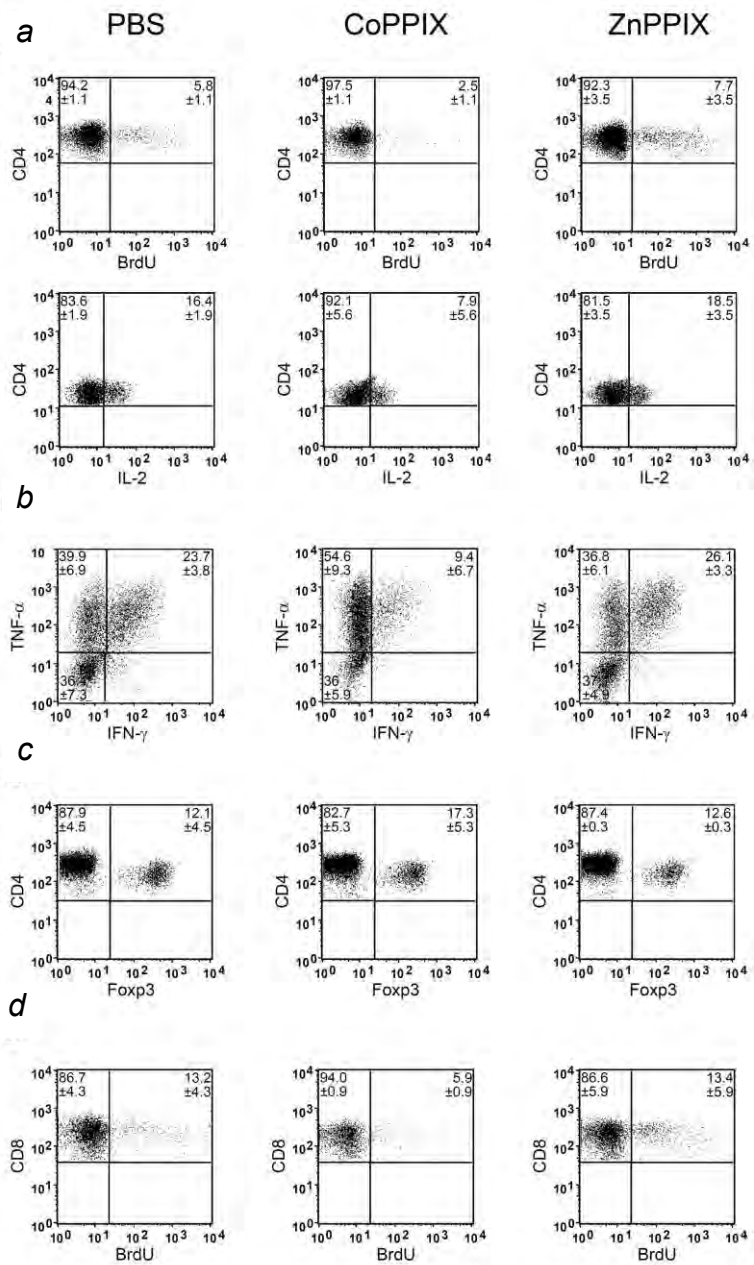
8. Supplementary Figures



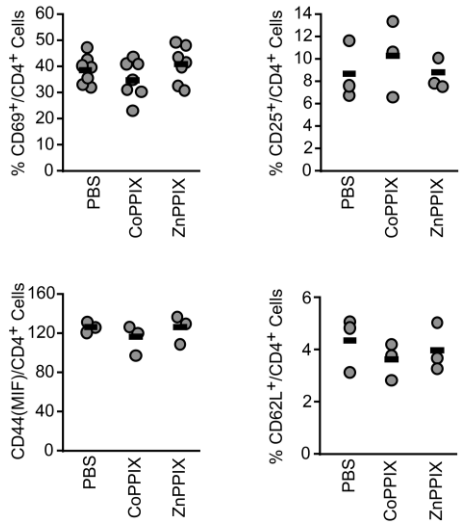
Supplementary Figure 1. HO-1 prevents CNS demyelination. Representative Luxol fast blue staining of spinal cord sections are shown for naïve C57BL/6 mice (***a***) versus C57BL/6 *Hmox-1*$^{+/+}$ (***b***) and *Hmox-1*$^{-/-}$ (***c***) mice, 60 days after EAE induction. Magnifications in (a-c) are 40x. Dashed rectangles in (a-c) are magnified (400x) in (*d*-f), respectively. Arrows indicate demyelination.



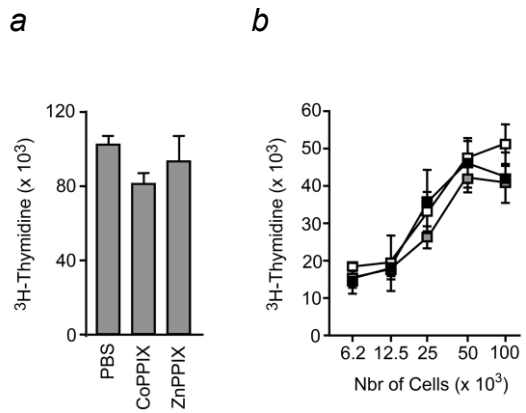
Supplementary Figure 2. CNS leukocyte infiltrates during EAE. Leukocyte infiltrates were analyzed by flow cytometry in (***a***) naïve C57BL/6 mice (n=2) or (***b***) 20 days after immunization. Representative plots are shown with relative percentages of CD45^{+}, CD11b^{+}, CD4^{+} and CD8^{+} cells ± standard deviation.



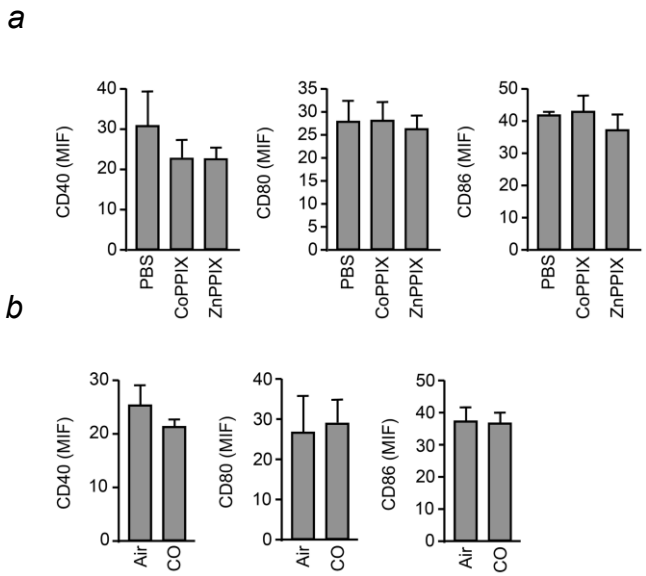
Supplementary Figure 3. HO-1 modulates T_H cell effector function within the CNS. EAE was induced in C57BL/6 mice, randomized two days after disease onset and treated daily with PBS, CoPPIX or ZnPPIX. CNS leukocyte infiltrates were analyzed by flow cytometry, 20 days post-immunization. When indicated, mice received BrdU. Representative plots are shown with mean percentages ± standard deviation (n=3-10 animals per staining). (***a***) Staining for intracellular BrdU *versus* surface CD4 and intracellular IL-2 *versus* surface CD4. (***b***) Staining for intracellular TNF-α *versus* intracellular IFN-γ is shown in $CD4^+$ T_H cells. (***c***) Staining for intracellular Foxp3 *versus* surface CD4. (***d***) Staining for intracellular BrdU *versus* surface CD8.



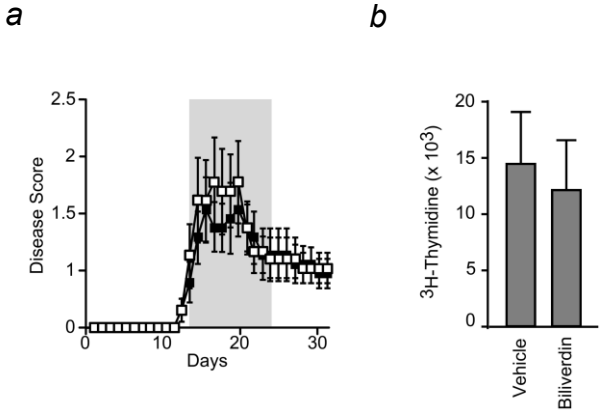
Supplementary Figure 4. Induction of HO-1 does not modulate the expression of activation markers in T_H cells within the CNS. EAE was induced in C57BL/6 mice, randomized two days after disease onset and treated daily with PBS, CoPPIX or ZnPPIX. Leukocyte infiltrates in the CNS were analyzed by flow cytometry, 20 days post-immunization. Each value represents an individual animal. Relative percentage of $CD69^+/CD4^+$ T_H cells, $CD25^+/CD4^+$ T_H cells and $CD62L^+/CD4^+$ T_H cells are shown. For CD44 the mean florescence intensity of CD44 is shown in $CD4^+$ T_H cells. Bars indicate mean value of all mice analyzed under each treatment.



Supplementary Figure 5. Induction of HO-1 does not suppress naïve myelin-reactive T_H cell priming. C57BL/6 mice were immunized in the footpad with MOG_{35-55} plus CFA and treated daily with PBS (n=3), CoPPIX (n=3) or ZnPPIX (n=3). Lymph node cells were isolated 8 days post-immunization. (***a***) T_H cell proliferation was measured *in vitro* by [^{3}H]-Thymidine incorporation, 72 hours after addition of concanavalin A (2 µg/ml). Results shown are the mean ± standard deviation of one representative assay out of five. (***b***) Increasing numbers of T_H cells (>98% $CD4^+$ T cells) from PBS- (white) (n=3), CoPPIX- (black) (n=3) or ZnPPIX- (grey) (n=3) treated mice were co-cultured with T_H cell-depleted lymph node cells (<98% $CD4^+$ T cells) from immunized but otherwise untreated mice. MOG_{35-55}-specific T_H cell proliferation was measured as in (a). Results shown are the mean ± standard deviation.



Supplementary Figure 6. Induction of HO-1 does not modulate the expression of costimulatory molecules in APC. C57BL/6 mice were immunized in the footpad with MOG_{35-55} plus CFA and (***a***) treated daily with PBS (n=4), CoPPIX (n=6), ZnPPIX (n=6),(***b***) exposed to air (n=6) or CO (n=7). Draining lymph node cells were isolated 8 days after immunization and CD40, CD80 or CD86 surface expression in DC ($CD11c^+$) was analyzed by flow cytometry. Quantifications (mean intensity of fluorescence; MIF) are shown as mean ± standard deviation.



Supplementary Figure 7. Biliverdin does not suppress EAE progression nor does it suppress the proliferation of myelin-reactive T_H cells. (***a***) EAE was induced, mice were randomized two days after disease onset and treated daily with biliverdin (black)(n=15) or vehicle (white)(n=15) for the period indicated by the shaded area. Daily clinical scores are shown as mean ± standard error of mean. (***b***) C57BL/6 mice were immunized in the footpad with MOG_{35-55} plus CFA and treated daily with biliverdin (n=3) or vehicle (n=3), starting two days prior to immunization. Draining lymph node cells were isolated 8 days post-immunization and myelin-reactive T_H cell proliferation was assessed *in vitro* by [^{3}H]-thymidine incorporation, 72 hours after addition of MOG_{35-55} (10 µg/ml). Results shown are the mean ± standard deviation from one assay with five independently treated animals per group.

Chapter 4

General Discussion

Immunity aims at protecting organisms from infection, clearing pathogens and thus, allowing a return to homeostasis[1]. Inflammation is an essential component of the immune response, triggered by the recognition of molecular patterns expressed by microbes or by molecules released upon tissue injury, by cellular or soluble PRR[2]. While inflammation aims at clearing the source of infection or noxious stimuli, it imposes a certain level of tissue damage. Therefore, mechanisms assuring tissue protection and/or repair are required for return to homeostasis. Tissue damage caused by oxidative stress, in particular, can be very deleterious as it may alter the function of macromolecules, lipids and proteins, causing mutations and epigenetic modifications, leading eventually to DNA damage[157]. This highlights the need for tight regulation of inflammatory and immune responses, as well as the type, magnitude and duration of the response mounted.

The first line of regulation acts on innate immune cells at the level of their activation, determining whether adaptive immunity will ensue. This is particularly relevant for DC that bridge innate and adaptive immunity and as such, play a central role in mounting protective as well as pathologic T_H cell responses, such as the one involved in the pathogenesis of autoimmune neuroinflammation[65].

The pathogenesis of autoimmune neuroinflammation is initiated by a break of peripheral tolerance, in which immunogenic DC activate naïve myelin-reactive T_H cells[252,253], followed by reactivation of primed T_H cells by perivascular DC in the CNS[254] and CNS invasion[65]. This leads to activation of resident CNS cells, recruitment of macrophages, T_H and T_C cells, among others, and establishes neuroinflammation[65]. CNS-infiltrating-macrophages and microglia become potent APC, boosting the T_H cell response and sustaining a pro-inflammatory env\ment in the CNS[255]. Damage caused to oligodendrocytes and neurons underlies the neurological symptoms of EAE, and presumably those of MS. In this context, mechanisms regulating DC immunogenicity, should contribute to restrain myelin-reactive T_H cell activation and as such, prevent the pathogenesis of autoimmune neuroinflammation. As demonstrated in chapter 2 the transcription factor Nrf2 seems to act in such a manner.

As previously shown[220,221], *Nrf2*-deficient (*Nrf2*$^{-/-}$) mice have an exacerbated EAE severity, when compared to *Nrf2*$^{+/+}$ mice in two different EAE models: active immunization of C57BL/6 mice with MOG_{35-55} in CFA (*Chapter 2 Fig.1b-d*) or PTx-induced EAE in 2D2 TCR transgenic C57BL/6 mice (*Chapter 2 Fig.1e-f*). This suggests that the regulatory effect of Nrf2 is not specific to a particular experimental model of EAE, but rather that it operates via a general mechanism of action with impact in autoimmune neuroinflammation. Pharmacologic activators of Nrf2, such as Dimethylfumarate, reduce the pathological outcome of EAE[221,222] and MS[223,224], suggesting that further understanding of the mechanism behind the protection afforded by Nrf2 in MS could be of high therapeutic value.

This protective mechanism is likely to rely on the expression of *Hmox1* (*Chapter 3*)[175], *Mt1 and Mt2*[256] genes regulated by Nrf2 that inhibit the pathogenesis of EAE. In keeping with this notion, these genes are up-regulated in active MS lesions as well as in the CNS of mice developing EAE[257] (*Chapter 2 Fig.1a* and *Suppl. Table1*). Interestingly, at steady state the level of expression of different *Nrf2*-dependent genes in the CNS varies considerably, i.e. *Fth* is expressed at very high levels when compared to any other gene analyzed (*Chapter 2 Suppl. Table1*). The reason for this is not understood, but it is known that oligodendrocytes accumulate Fe in the cytoplasm[258]. As labile Fe can be deleterious, acting as a potent pro-oxidant via the Fenton chemistry[209], it is likely to be stored safely under a less reactive form in the cytoplasm, when bound to FtH[259]. In addition, oligodendrocytes can uptake Fe via soluble FtH[260], that binds extracellular Fe and transports it via the T cell immunoglobulin and mucin domain-containing protein-2 receptor into the cytoplasm[261]. The dependency on FtH for Fe uptake and storage in oligodendrocytes could explain the high levels of expression of this protein observed in the CNS of naïve mice (*Chapter 2 Suppl. Table1*).

Nrf2 is activated by changes in the cellular redox status and restores homeostasis by up-regulating a set of stress-responsive genes[169]. Activation of Nrf2 has cytoprotective properties via the expression of genes that help cells cope with the damaging effects of oxidative stress[162]. The environment generated in a tissue undergoing inflammation, such as the CNS during EAE or MS, contains high levels

of ROS and RNS that can cause damage to oligodendrocytes and neurons[157,163]. Hence, activation of the Nrf2 pathway in the CNS should limit the deleterious effects of oxidative stress, presumably ameliorating EAE, by providing tissue-damage control. Surprisingly however, the mechanism underlying the salutary effect of Nrf2 against autoimmune neuroinflammation[220,221] (*Chapter 2 Fig.1b-g*) does not involve cellular adaptation to oxidative stress in resident cells of the CNS (*Chapter 2 Fig.2d-f*).

Another possible mechanism for the protective effect of Nrf2 against autoimmune neuroinflammation might rely on its ability to regulate the expression of pro-inflammatory mediators by innate immune cells[170]. This has been previously suggested for several experimental models of inflammatory diseases, including EAE[220]. Higher EAE severity observed in *Nrf2*$^{-/-}$ *vs*. *Nrf2*$^{+/+}$ mice in the Biozzi genetic background, is associated with increased expression of mRNA encoding cytokines (*Il1b*, *Tnf*, *Il12* and *Ifng*) and chemokines (*Cxcl9* and *Cxcl13*) in the CNS at the onset of EAE[220]. However, in our study, cytokine and chemokine expression in the CNS was similar at the onset of EAE in *Nrf2*$^{-/-}$ *vs*. *Nrf2*$^{+/+}$ C57BL/6 mice (*Chapter 2 Suppl. Fig.3*). This apparent discrepancy may reflect differences in EAE progression in the two experimental models. While Nrf2 deletion in the Biozzi genetic background is associated with increased EAE severity at the onset of disease, in the C57BL/6 genetic background this only becomes apparent at later stages of disease progression. Thus, it is conceivable that cytokine and chemokine expression in the CNS would be observed at later stages of disease progression in C57BL/6 mice lacking Nrf2. However, due to the high rate of mortality of *Nrf2*$^{-/-}$ *vs*. *Nrf2*$^{+/+}$ C57BL/6 mice (*Chapter 2 Fig.1d*) this hypothesis is difficult to test experimentally.

Our data suggests that Nrf2 controls IL-12 production by DC and, therefore, restrains the priming of myelin-reactive effector T_H1 cells, involved in the pathogenesis of autoimmune neuroinflammation. This is suggested by the following observations. First, when transferred into syngeneic recipients, leukocytes from MOG_{35-55}-immunized *Nrf2*$^{-/-}$ mice elicit a more severe form of EAE, as compared to *Nrf2*$^{+/+}$ leukocytes (*Chapter 2 Fig.2a-c*). The reverse is not true, in that adoptive

transfer of leukocytes from MOG_{35-55}-immunized mice elicit similar EAE severity in wild type *Nrf2*$^{+/+}$ *vs. Nrf2*$^{-/-}$ mice (*Chapter 2 Fig.2d-f*). The adoptively transferred leukocytes are not purified activated T_H cells, and therefore, we cannot exclude that *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ APC are also transferred, but it is unlikely that these cells would play a role in T_H cell re-stimulation in the CNS of recipient mice as their turnover *in vivo* is approximately 3 days[262]. Nrf2 expression in DC does not influence T_H cell proliferation (*Chapter 2 Fig.3a,b,d*), but does impact on effector T_H cytokine production (*Chapter 2 Fig.3c,e*). As the instruction for T_H cell lineage differentiation is provided during DC/T_H cell priming, and not upon T_H cell reactivation[28], this supports further the notion that T_H cell priming is the process targeted by Nrf2.

Depending on the experimental T_H cell priming system analyzed, i.e. *in vivo* or *in vitro*, Nrf2 expression can regulate the differentiation of T_H1 (IFN-γ), T_H17 (IL-17A) and T_H2 (IL-13) (*Chapter 2 Fig.3c*) or just of T_H1 cells (*Chapter 2 Fig.6a*), respectively. The apparent discrepancy between these two experimental systems probably reflects the relative contributions of different cell populations for T_H cell priming *in vivo* or *in vitro*. While T_H cell priming is controlled by DC *in vitro*, other cells can influence T_H cell priming *in vivo*.

DC-derived IL-12 is necessary and sufficient to drive naïve T_H cell differentiation towards the T_H1 lineage[44], while the involvement of DC-derived cytokines for T_H17 or T_H2 cell differentiation is still a matter of debate[20]. T_H17 differentiation requires IL-6 and TGF-β[37], while T_H2 priming is dependent on IL-4[37]. TGF-β[263] is produced in an autocrin manner by T_H cells, while IL-6 can be produced by innate immune cells, other than DC[264]. The cellular source of IL-4, involved in T_H2 differentiation, is still controversial[265-267]. Thus, it is possible that other immune cell populations producing high levels of IL-6 and IL-4 are responsible for the increased production of IL-17A and IL-13 by *Nrf2*$^{-/-}$ T_H cells *in vivo* (*Chapter 2 Fig.3c*), while IL-12 production by DC accounts for the increase in IFN-γ both *in vitro* and *in vivo* (*Chapter 2 Fig.3c* and *6a*).

Regulation of T_H1 differentiation by Nrf2 is determined by the expression of this transcription factor in DC (*Chapter 2 Fig.6a*), suppressing IL-12 (*Chapter 2*

Fig.5d), whilst promoting IL-6 and TNF production (*Chapter 2 Fig.5e,f*). This effect should inhibit the differentiation of naïve myelin-reactive T_H cells towards a T_H1 phenotype, and possibly promote T_H17 differentiation. This appears to be the case for T_H1 cells, as demonstrated by the observation that blocking IL-12/23p40 *in vitro* is sufficient to revert the T_H1 bias observed when Nrf2 expression is absent in DC (*Chapter 2 Fig.7a*). However, the observation that Nrf2 does not regulate the number of IFN-γ-producing T_H1- or T_H17-infiltrating cells in the CNS of mice undergoing EAE is difficult to conciliate with this notion (*Chapter 2 Suppl. Fig.1* and *2*). Several factors can account for this observation. First, re-stimulation of CNS-infiltrating leukocytes *in vitro* with PMA and ionomycin, as performed in our study, can promote cytokine production in a manner that does not reflect the profile of cytokine production by effector T_H cell subsets *in vivo*[134]. This can be bypassed by antigen-specific re-stimulation of CNS-infiltrating leukocytes *in vitro* or using anti-CD3 mAb, which reflect more faithfully the cytokine production of effector CNS-infiltrating T_H cells *in vivo*[134].

It is possible as well that this apparent discrepancy may reflect a kinetic issue, as CNS-infiltrating T_H17 cells stop secreting IL-17 and convert to IFN-γ-producing T_H cells, becoming the major source of IFN-γ during the late phase of EAE[134]. Therefore, elevated levels of IFN-γ, observed in *in vivo* and *in vitro* priming systems (*Chapter 2 Fig.3c* and *6a*), may be masked during EAE by the conversion of T_H17 cells to IFN-γ-producing cells in the CNS. This should not negate that IFN-γ-producing T_H1 cells play a crucial role in the pathogenesis of EAE.

That the ability of Nrf2 to control the production of IL-12, and subsequently the IFN-γ production by myelin-reactive T_H1 cells contributes to the protective effect of Nrf2 is revealed by the observation that inhibition of IL-12/23p40 (*Chapter 2 Fig.7c-e*) or IFN-γ (*Chapter 2 Fig.6c-e*) signaling reduces EAE severity in *Nrf2*-deficient mice. IFN-γ is a pleiotropic cytokine that exerts immunomodulatory effects in a variety of immune cells[268]. These comprise: i) up-regulation of MHC class I and II expression in APC and endothelial cells; ii) up-regulation of adhesion molecule expression and chemokine secretion by endothelial cells; iii) priming of innate

immune cells for the production of pro-inflammatory cytokines, such as IL-1 and TNF and iv) the activation and proliferation of T_C cells[268,269]. However, a number of immunosuppressive functions have also been assigned to IFN-γ, including: i) inhibition of cell proliferation; ii) induction of T_H programmed cell death, iii) inhibition of T_H2 and T_H17 cell differentiation and iv) conversion of activated T_H cells into T_{REG}[268-270]. Possibly, as a consequence of this wide range of effects, the results obtained when studying the role of IFN-γ in EAE have been difficult to interpret and conciliate with the evidence that IFN-γ-producing T_H1 cells are pathogenic in EAE. Despite this controversy, it is clearly established that signaling by T_H1-derived IFN-γ is necessary and sufficient to determine the localization of the lesions within different anatomical compartments of the CNS[92,271]. The pathogenic effect of IFN-γ produced by T_H1 cells acts selectively in the spinal cord while protective in the brain, brainstem and cerebellum, as revealed by adoptive transfer of wild type encephalitogenic T_H1 cells into *Ifngr*$^{-/-}$ recipients or *Ifng*$^{-/-}$ T_H1 cells into wild type recipients. In both cases, adoptive transfer results in the development of atypical EAE, with prominent inflammatory infiltrates in the cerebellum and the brain, but not in the spinal cord[271].

We propose a mechanism of action underlying the protective effect of Nrf2 that is somehow distinct from the one proposed by previous studies. While we propose that Nrf2 controls IL-12 expression in DC, thus inhibiting T_H1 differentiation, other studies have suggested that Nrf2 expression in DC promotes T_H2 responses or reverses T_H1-reduced immunity.

Exposure of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ DC to urban ambient particulate matter (APM), a T_H2-inducing stimuli, suggested that Nrf2 promotes a T_H2-type immune response[272]. This study used GM-CSF-derived bone marrow or pulmonary DC while we used splenic DC in our study. This would suggest that Nrf2 may have different effects in specific DC populations, and presumably therefore act in a tissue-specific manner. Another difference between this study and ours relates to the inflammatory agonist used to render DC immunogenic, APM *vs.* LPS, CpG and Poly(I:C). This would suggest that expression of Nrf2 in DC affects T_H cell priming in a stimulus-

specific manner. Presumably, when *Nrf2*$^{-/-}$ DC are stimulated with a T_H1 inducer, such as the TLR4 agonist, LPS, a heightened T_H1 response is to be expected and so is increased severity of T_H1-driven pathology, such as the development of EAE in response to MOG_{35-55} plus CFA immunization. Nevertheless, when *Nrf2*$^{-/-}$ DC are exposed to T_H2 inducers, such as APM, the T_H2 response should be enhanced and T_H2-driven pathology worsened, as demonstrated for lung inflammation. In both cases however, Nrf2 down-modulates T_H cell responses by controlling DC immunogenicity in a stimulus-dependent manner. In agreement with this, Nrf2 does not appear to act on T_H cells to control differentiation of specific T_H cell lineages.

Another study revealed that DC treated with compounds that activate Nrf2 reverse the decreased T_H1 immunity observed in aged mice[273]. However, this study cannot be directly compared to ours since again GM-CSF-derived bone marrow DC were used, while splenic DC were used in ours. In addition, our study used 2-month old mice, while this study used 20-month old mice. Importantly, aged *Nrf2*$^{-/-}$ mice have been described to develop several immune-mediated diseases, such as systemic lupus erythrematosus[213,214] and immune-mediated hemolytic anemia[215] and as such, the homeostatic state of DC from mice in this study and ours might be different.

The molecular mechanism via which Nrf2 controls the induction of IL-12 expression in DC, in response to PRR signaling, is not clear at the moment. One possibility is that this mechanism would act in a cell-autonomous manner via the transcriptional repression of different *Il12* gene family members. This notion is supported by the observation that induction of *Il12/23p40* (*Chapter 2 Fig.5a*) and *Il12p35* (*Chapter 2 Fig.5c*) mRNA expression in response to the TLR4 agonist, LPS, is increased in DC lacking *Nrf2*, as compared to wild type DC. Also in keeping with this notion is the finding that Nrf2 inhibits the activation of several transcription factors regulating the expression of different *Il12* gene family members[173,225], including NF-κB and IRF-3, as demonstrated for TLR4 signaling in mouse embryonic fibroblasts[217]. A similar mechanism could operate in DC given that induction of IL-12/23p40 and IL-12p35 expression in response to LPS is driven by a mechanism dependent on NF-κB activation as well as the activation of different IRF

family members[274], including IRF-1, -3 and -7 that regulate IL-12p35 expression, and IRF-5 that regulates IL-12/23p40 expression or IRF-8 that regulates both IL-12p35 and IL-12p40 expression[274]. The mechanism by which Nrf2 would regulate the activation of these transcription factors in DC remains unclear, although it has been suggested to rely on the regulation of the cellular redox status[217], which can modulate NF-κB activation in response to LPS[275,276]. In keeping with this notion, expression of anti-oxidant genes is impaired in *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ DC (*Chapter 2 Fig.4e-i*), probably explaining why there is an accumulation of free radicals and a redox imbalance in *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ DC[277], possibly promoting IL-12 expression (*Chapter 2 Fig.5a,c*) via NF-κB activation. Nevertheless, induction of *Il6*[278] and *Tnf*[279] mRNA expression in response to LPS is also regulated by NF-κB, but the expression of these genes is reduced in *Nrf2*$^{-/-}$ *vs. Nrf2*$^{+/+}$ DC, arguing for a specific mechanism regulating the transcription of *Il12* gene family members.

The increased levels of IL-12p40 observed in *Nrf2*$^{-/-}$ DC (*Chapter 2 Fig.5b*), compared to wild type DC, in response to TLR3 (Poly(I:C)) stimulation could be the result of a combined effect of Nrf2 on NF-κB and IRF-3 activation. This is suggested by the experimental evidence that Nrf2 also inhibits IRF-3 activity[217].

An alternative hypothesis is that Nrf2 inhibits *Il12p35* and *Il12/23p40* expression via directly binding to ARE in the promoter regions of these genes. Both genes have sequences of putative ARE in their promoters, where Nrf2 could bind (*Ana Cunha, unpublished observations*). Nevertheless, Nrf2 is a transcriptional activator and there is only one study suggesting that a caspase-3-cleaved form of Nrf2 can act as a negative regulator of gene transcription[280], pointing to the unlikeliness of this mechanism.

Another possible explanation is that Nrf2 regulates de ontogeny of DC subsets and thus, the composition of splenic DC subsets, that may differ between *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice. Splenic DC subpopulations have different abilities to promote the differentiation of distinct T_H cell lineages. CD8α^+ DC express high levels of CD70 and secrete IL-12, stimulating T_H1 responses upon activation, in contrast to CD8α^- DC, which express low levels of CD70 and do not produce

significant levels of IL-12, inducing T_H2 responses[45,47,48,281]. It is possible, therefore, that there is an over-representation of CD8α^+ DC in the spleen of *Nrf2*$^{-/-}$ mice, when compared to *Nrf2*$^{+/+}$ controls. However, analysis of DC subpopulation composition in Flt3L-derived bone marrow DC, which are thought to resemble splenic DC-derived subsets[26], revealed a similar profile between *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice (*Ivo Marguti, unpublished observations*), suggesting Nrf2 does not regulate DC subset ontogeny. However, this should be further confirmed by comparing splenic DC subpopulation composition in *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice.

The molecular mechanism via which Nrf2 controls the expression of IL-12 in DC might rely also on the expression of Nrf2-responsive genes that limit the accumulation of ROS in innate immune cells[217]. This notion is supported by the observation that Nrf2 can inhibit the accumulation of ROS in a variety of cell types, including DC[277]. *Hmox1* is one of such genes (*Chapter 2 Fig.4i*), a notion supported by the observation that when exposed to LPS *in vitro*, *Hmox1*$^{-/-}$ DC produce higher levels of IL-12, as compared to *Hmox1*$^{+/+}$ DC (*Chapter 2 Fig.8a*). This suggests that Nrf2 controls the expression of IL-12 via a mechanism involving the expression of HO-1 in DC. This notion is also in keeping with the observation that exogenous delivery of CO, a by-product of HO-1 activity, can inhibit the expression of IL-12 in DC[282]. Moreover, a recent study has shown that treatment of DC with an inducer of the Nrf2 pathway, Dimethylfumarate, reduces the expression of *Il12* gene family members in DC via a mechanism that involves inhibition of STAT-1 phosphorylation and induction of HO-1 expression[222]. Silencing of STAT-1 signaling suppresses the expression of *Il12p35* mRNA, via a mechanism involving IRF-8 inhibition, while HO-1 induction inhibits the transcription of *Il12/23p40* and *Il23p19*[222]. Inhibition of IL-23p19 expression relies on the interaction of a nuclear N-terminal truncated portion of HO-1 with NF-κB binding sites on the *Il23p19* promoter, thus inhibiting its transcription[222]. This raises the possibility that HO-1 inhibits the expression of IL-12/23p40 via the same mechanism, which would be consistent with the observation that increased *Il12/23p40* expression in *Nrf2*-deficient DC (*Chapter 2 Fig.5a*) is associated with suppression of HO-1 expression (*Chapter 2 Fig.4i*), as compared to wild type DC. As for the regulation of IL-12p35, it has never been addressed

whether STAT-1 phosphorylation would be increased in *Nrf2*-deficient cells, but this is a possibility that could explain why *Nrf2*$^{-/-}$ DC produce more *Il12p35* (*Chapter 2 Fig.5c*), as compared to DC that express Nrf2.

Expression of HO-1 in DC fails to inhibit the differentiation of naïve T_H cells towards the T_H1 lineage (*Chapter 2 Fig.8c*), suggesting this effect is mediated probably via other Nrf2-responsive genes. In addition, the overall protective effect of Nrf2 against EAE does not appear to act via a mechanism involving the expression of HO-1 in DC. This notion is supported by the observation that specific down-regulation of the *Hmox1* allele in DC has no impact on the pathological outcome of EAE (*Chapter 2 Fig.8d-f*). This suggests that expression of Nrf2 in DC confers protection against EAE via a mechanism driven by the expression of HO-1 in combination with other Nrf2-responsive genes. The identity of these genes remains elusive at the moment, but Metallothioneins (*Mt1* and *Mt2*) are possible candidates, as they are Nrf2-regulated genes with a demonstrated role in the inhibition of the pathological outcome of EAE[256].

Several studies have suggested that HO-1 inhibits DC maturation, exerting immunosuppressive effects that limit the pathological outcome of immune-mediated inflammatory diseases[185], including autoimmune neuroinflammation[174,175]. It should be noted, however, that most of the studies addressing the ability of HO-1 to modulate DC maturation are based on pharmacologic modulation of HO-1[174,282,283]. Given that the pharmacologic molecules used in these studies can inhibit DC maturation irrespectively of HO-1[284], the physiologic relevance of these findings is difficult to assess. This notion is supported by the observation that, when expressed under physiologic conditions, HO-1 fails to regulate DC maturation (*Chapter 2 Suppl. Fig.14* and *15* and *Appendix 1 Fig.3*) as well as T cell proliferation (*Chapter 2 Fig.8b*). Although, expression of HO-1 appears to exert immunoregulatory effects in DC, i.e. inhibition of IL-12 production (*Chapter 2 Fig.8a*), these effects do not seem to be sufficient to suppress the pathogenesis of autoimmune neuroinflammation (*Chapter 2 Fig.8d-f*).

Although DC-specific deletion of *Hmox1* does not modulate the pathogenesis of EAE, systemic (*Chapter 3 Fig.1a* and *Table 1*) or myeloid-specific

ablation[175] have been shown to do so. This suggests that the protective effect of HO-1 expression relies on an immunoregulatory effect exerted by myeloid cells, other than DC. HO-1 was shown to form a complex with IRF-3 and to be essential for IRF-3 activation and subsequent gene expression in response to TLR3/TLR4 signaling[175]. *Hmox1* deletion leads to defective IFN-β production, and thus, increased activation of macrophages and accumulation of T_H1 and T_H17 cells in the CNS[175], causing an exacerbated form of EAE. Moreover, IFN-β has therapeutic effects in EAE and MS[285]. Therefore, by promoting IFN-β production in myeloid cells, HO-1 might limit the progression of autoimmune neuroinflammation.

Pharmacological induction of HO-1 expression (*Chapter 3 Fig.1d*), after the onset of clinical disease, suppresses EAE progression (*Chapter 3 Fig.1b,c* and *Table2*). This effect is independent of the mouse genetic background or peptide used to induce EAE (*Chapter 3 Fig.1b,c*), arguing for a general regulatory mechanism. This mechanism requires the expression of HO-1 (*Chapter 3 Fig.1e*) and presumably involves HO-1 activity, as suggested by the observation that exogenous CO mimics in part the protective effects of HO-1 (*Chapter 3 Fig.1f*). However, the protective effect of CO is more limited than that of pharmacological induction of HO-1, suggesting that other end products of heme catabolism by HO-1, i.e. biliverdin and/or Fe, might also contribute to this effect.

Beneficial effects of biliverdin reductase[286] and bilirubin[239] administration have been reported in the context of EAE. Billirubin is a lipophilic antioxidant[239], being oxidized to biliverdin, with biliverdin reductase sustaining an amplification cycle whereby biliverdin is recycled back into bilirubin[286]. However, we did not observe a salutary effect of biliverdin administration during EAE (*Chapter 3 Suppl. Fig.7*). A possible explanation for this might be that biliverdin does not have the same antioxidant power as bilirubin and/or cannot gain access to the CNS parenchyma. Thus, bilirubin inhibits EAE possibly due its ability to cross the BBB, acting as an antioxidant in the CNS.

It is also possible that the protective effect of HO-1 against EAE operates indirectly via a reduction of the cellular labile Fe pool, thereby limiting its availability

to participate in the Fenton chemistry, via the up-regulation of FtH[211] and Fe efflux pumps[210]. Several lines of evidence support a pathogenic role of Fe in EAE. First, Fe deposits are present in lesions of individuals with MS and in the CNS of mice with EAE[243,258,287]. Second, mice fed a low-Fe diet do not develop EAE[288]. Third, pharmacological Fe chelation reduces EAE severity[289]. Fe is localized mainly to the cytoplasm of oligodendrocytes bound to ferritin (*Chapter 2 Fig.1a*) and transferrin[259] and therefore, oligodendrocyte injury may release Fe, increasing the labile Fe pool during EAE and MS. This hypothesis remains to be formally addressed.

Several lines of evidence suggest HO-1 modulates T cell reactivation and effector function, presumably via regulation of antigen presentation by APC. Pharmacological induction of HO-1 is associated with inhibition of both T_H1 and T_C cell accumulation, proliferation and IFN-γ production within the CNS (*Chapter 3 Fig.3*). Since T_H1 and T_C cells are effectors of damage in the CNS during EAE, limiting T_H1 and T_C CNS-accumulation, might limit CNS damage. It remains to be tested if pharmacological induction of HO-1 affects the development and/or function of T_H17 cells.

Pharmacological induction of HO-1 is associated with inhibition of T_H cell proliferation *in vitro* via a mechanism mediated by APC (*Chapter 3 Fig.4f*). This mechanism does not influence T_H cell priming, while limiting T_H cell reactivation (*Chapter 3 Fig.4e* and *Suppl. Fig.5*). Within the APC compartment, DC are sufficient to mediate this effect (*Chapter 3 Fig.4g*). However, a possible contribution of macrophages has not been discarded. During EAE, perivascular DC are required to reactivate primed encephalitogenic T_H cells, allowing CNS invasion[254], while CNS-resident APC reactivate T_H cells at later stages once access to the CNS is facilitated[255]. Importantly, pharmacological induction of HO-1 induces expression of HO-1 in CNS-associated DC (*Chapter 3 Fig.5a*). Taking this into account, pharmacological induction of HO-1 might inhibit the reactivation of encephalitogenic T_H cells, via modulation of DC activation, and impact on the initial events leading to the establishment of neuroinflammation. Furthermore, by modulating microglia/macrophage activation, HO-1 would counter T_H cell reactivation and the inflammatory milieu established in the CNS. In keeping with this notion, down-

modulation of encephalitogenic T_H cell activation and effector function (*Chapter 3 Fig.3*), by pharmacological induction of HO-1 is associated with inhibition of MHC class II expression in APC, including DC, infiltrating-Mø and microglia (*Chapter 3 Fig.6a,c,d*). Expression of MHC class II by DC is of central importance for the activation of naïve T_H cells and generation of T_H responses, such as the one involved in the pathogenesis of EAE[65]. In addition, MHC class II, expressed on CNS APC is crucial for T_H cell reactivation within the CNS[254,290].

Expression of MHC class II is regulated mainly at the transcriptional level[291] by nuclear transcription factor Y , CREB and regulatory factor X, that bind consensus DNA sequences in the MHC class II gene promoter, forming a transcriptionally inactive multiprotein complex[291]. Recruitment of class II transactivator (CIITA) is required to activate MHC class II gene transcription by interacting with the basic transcriptional machinery, as well as by altering chromatin conformation[291,292]. CIITA acts as a master regulator of MHC class II expression determining whether or not cells express MHC class II[293].

Induction of MHC class II in response to IFN-γ is strictly dependent on CIITA[293,294]. Ligation of the IFN-γ receptor triggers a signal transduction pathway that activates the Janus kinase (JAK) family, phosphorylating/activating STAT-1[295] that dimerizes and translocates to the nucleus where it binds the CIITA promoter and activates its transcription[296]. Pharmacological induction of HO-1 inhibits STAT-1 activation and is associated with a reduction in CIITA expression in microglial cells (*Chapter 3 Fig.7c,d*), an effect likely to explain the inhibition of MHC class II expression observed in these cells *(Chapter 3 Fig.6c*). The molecular mechanism via which HO-1 or its by-products inhibit STAT-1 phosphorylation remains to be established. One study suggests that CO can modulate STAT-1 activation by phosphatidylinositol 3-kinase/Akt and p38 MAPK signaling pathways in endothelial cells. Whether, the same is true for JAK in APC remains to be tested[297].

Presumably, regulation of CIITA, and consequently of MHC class II expression by modulation of STAT1 by HO-1 or CO is not likely to act on DC since expression of MHC class II in DC appears to be independent of STAT-1[298]. Nevertheless, down-modulation of MHC class II might limit T_H clonal expansion and

differentiation towards an encephalitogenic phenotype. However, this effect has been shown to require activation of STAT-3, and to act irrespectively of HO-1[284]. This suggests that the protective effects associated with pharmacologic induction of HO-1 in EAE could be the result of a combined effect of HO-1-independent, STAT-3-mediated effects in DC, reducing T_H cell reactivation and CNS invasion, and of HO-1-dependent mechanisms inhibiting STAT-1 phosphorylation and CIITA expression in infiltrating-macrophages/microglia, thus limiting T_H cell reactivation within the CNS and preventing the consequences of autoimmune neuroinflammation.

Mice with systemic *Hmox1* deletion do not show increased expression of MHC class II in DC (*Appendix 1 Fig.3b,g*), macrophages or microglia (*Appendix 1 Fig3b,h and 4c,d,g,h*) or increased T cell effector cytokine production (*Appendix 1 Fig.5b,c,f,g and 6b,c,f,g and 7c,d,g,h*). This reveals that enforcing pharmacologically HO-1 expression at the onset of disease, although salutary, has distinct effects to those associated with physiological HO-1 expression during EAE. Nevertheless, these effects could be of important clinical relevance.

The involvement of T_{REG}, a T cell subset critically involved in the control of autoimmune and inflammatory responses[32,299], in the salutary effects of Nrf2 and/or HO-1 also remains to be tested. We found that neither *Nrf2* or *Hmox1* deletion, nor pharmacological induction of HO-1 expression, influenced the frequency or number of T_{REG} cells within the CNS of mice undergoing EAE, suggesting that the protective effect of HO-1 and Nrf2 does not act via T_{REG} modulation (*Chapter 2 Suppl. Fig.1h* and *2h* and *Chapter 3 Fig.3b*). However, the possibility of a functional interplay between Nrf2 and/or HO-1 and T_{REG} activity in EAE cannot be excluded. Several observations suggest that T_{REG} can affect DC function[300,301], indicating that the salutary effects afforded by T_{REG} can be mediated, at least partially by the modulation of APC activity. As such, a possible scenario would be that under inflammatory conditions T_{REG} would up-regulate the expression of Nrf2 and/or HO-1 in APC, modulating their activity and controlling T_H cell activation. Nonetheless, the impact of Nrf2 on T_{REG} function has to the best of our knowledge never been analyzed in detail. While it has been suggested that HO-1 expression in DC[302]

and/or T_{REG}[303,304] can regulate T_{REG} function[305,306], under homeostatic conditions the number and function of T_{REG} is similar between *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ mice. However, it remains to be tested if the same would be the case under inflammatory conditions *in vivo*, such as during EAE and MS.

In conclusion, this Thesis reveals that Nrf2 expression and pharmacological induction of HO-1 play a previously underappreciated role in innate immune cell regulation, with impact on the T_H cell response at the core of autoimmune neuroinflammation. First, we demonstrate that Nrf2 down-regulates the expression of IL-12 in DC, limiting T_H1 lineage differentiation and thus modulating the severity of autoimmune neuroinflammation. Second, we found that one of the genes regulated by the Nrf2 pathway, *Hmox1* controls the severity of EAE and its pharmacological induction inhibits the expression of MHC class II in APC and thus, the T cell response and severity of EAE. Presumably, this effect is mediated via a mechanism involving the production of CO. Altogether these data reveals the importance of Nrf2 and HO-1 in the control of autoimmune neuroinflammation, via the modulation of APC and consequently of adaptive immunity.

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Appendix 1

1. Introduction

We have demonstrated that *Hmox1*-deficient mice (*Hmox1*$^{-/-}$) mice develop a more severe form of EAE than wild type (*Hmox1*$^{+/+}$) animals (*Chapter 3 Fig.1a*). We have also shown that pharmacological induction of HO-1, at the onset of disease, reduces EAE severity in a HO-1-dependent manner (*Chapter 3 Fig.1b,c,e*). This salutary effect is associated with a reduction of MHC class II expression in APC and down-regulation of T_H cell reactivation, proliferation and effector function (*Chapter 3 Fig.3* and *Fig.4*). This raised the hypothesis that HO-1 might down-regulate the immune response causing EAE, by acting on APC. To gain more understanding on the physiologic role of HO-1 during EAE we characterized the: 1) profile of HO-1 expression in the CNS and 2) the immune response of *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ mice undergoing EAE.

2. Results

2.1. HO-1 is expressed in microglia/macrophages and astrocytes of mice with EAE.

HO-1 had been previously reported to be expressed in lesions of individuals with MS and in the CNS during EAE, specifically in infiltrating-macrophages/microglia and astrocytes[1]. In addition, we have demonstrated that HO-1 is also expressed in CNS-resident DC (*Chapter 3 Fig.5a*). To gain further insight into the cellular pattern of expression of HO-1 in the CNS, we performed double immunofluorescence stainings of HO-1 and CNS cell-specific markers in spinal cord sections of mice at the remission phase of EAE. This time point was chosen based on the observations that: 1) HO-1 is expressed in this time-point during EAE[2] and 2) *Hmox1*$^{-/-}$ mice undergoing EAE fail to remit, consistent with the notion that HO-1 plays a role and thus, is expressed at this time point. As others had reported, we observed that HO-1 expression in the spinal cord is mostly associated with the sites of lesion, where abundant cell infiltration is present (*Fig1a-d*). In addition, HO-1 expression could be detected in infiltrating-macrophages or microglia, as assessed by the expression of CD11b (*Fig.1b-g*) or F4/80 (*Fig.1h-j*) and astrocytes, as assessed by GFAP

expression (*Fig.1k-m*). HO-1 expression was not detected in endothelial cells, using CD31 as a marker, or in oligodendrocytes, stained with CNPase (data not shown). This suggests that HO-1 could play a regulatory role not only in infiltrating-immune cells but also in CNS-resident cells.

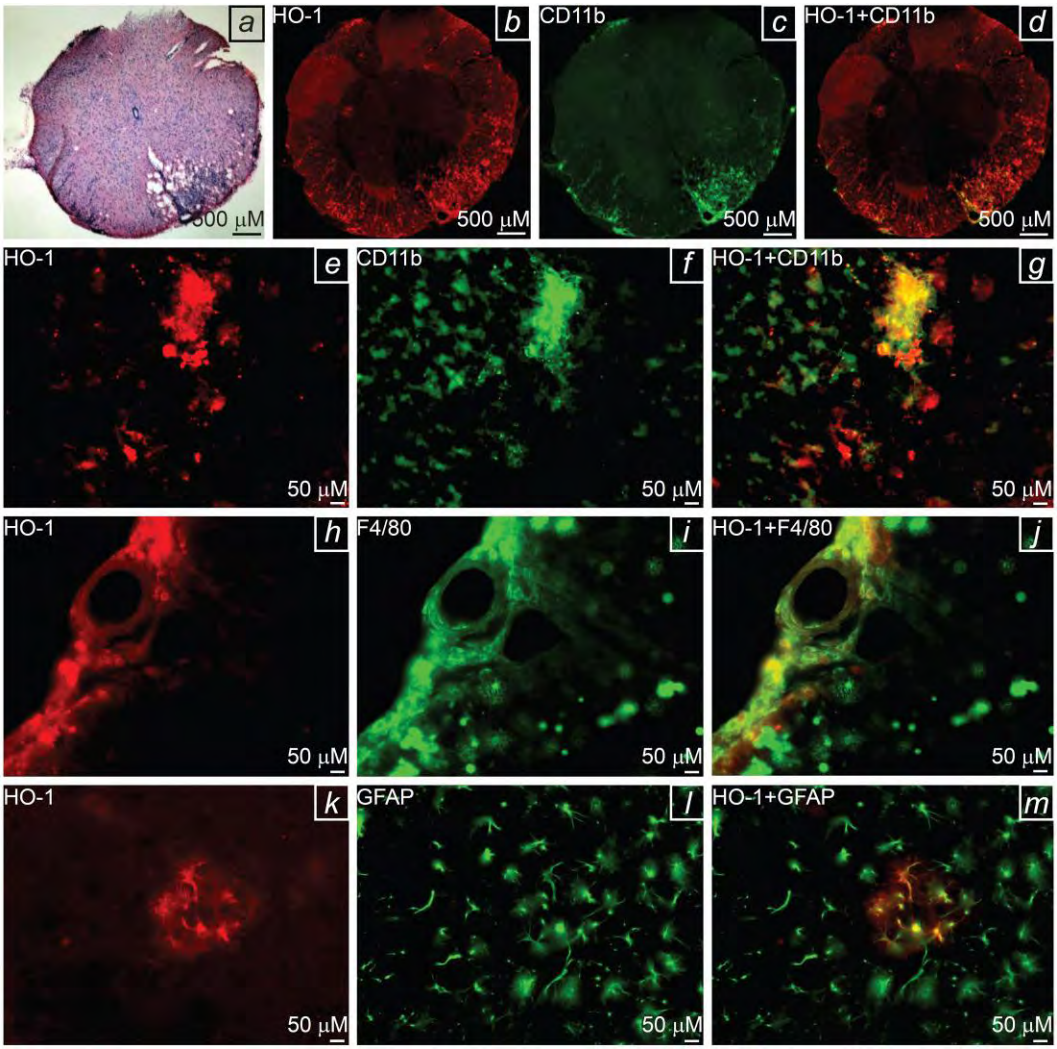

Figure 1. HO-1 expression in cells of the CNS during EAE. Immunostaining of (***a,e,h,k***) HO-1 in PFA-fixed CNS sections, of a C57BL/6 mouse immunized with MOG_{35-55} in the remission phase, and double-labeled with (***c,f***) CD11b, (***i***) F4/80 and (***l***) GFAP. Overlay of HO-1 and (***d,g***) CD11b, (***j***) F4/80 or (***m***) GFAP. Data shown is from one mouse. Magnifications: (a-d) 4x and (e-m) 40x.

2.2. HO-1 deletion does not impact in a relevant manner the immune response ongoing during EAE.

We analyzed by flow cytometry innate and adaptive immune cell populations and their activation status both in the periphery and in the CNS of *Hmox1*$^{-/-}$ *vs.* *Hmox1*$^{+/+}$ mice at the peak and remission of EAE. At the peak of EAE the total number of splenocytes was increased in *Hmox1*$^{-/-}$ *vs*. *Hmox1*$^{+/+}$ mice (*Fig.2a*), but this was no longer observed in the remission phase (*Fig.2c*). It should be noted that naïve *Hmox1*$^{-/-}$ mice develop splenomegaly, characterized by increased cellularity[3-5], which might account for this observation. Importantly this did not lead to an increased accumulation of leukocytes in the CNS of *Hmox1*$^{-/-}$ vs. *Hmox1*$^{+/+}$ mice (*Fig.2b,d*).

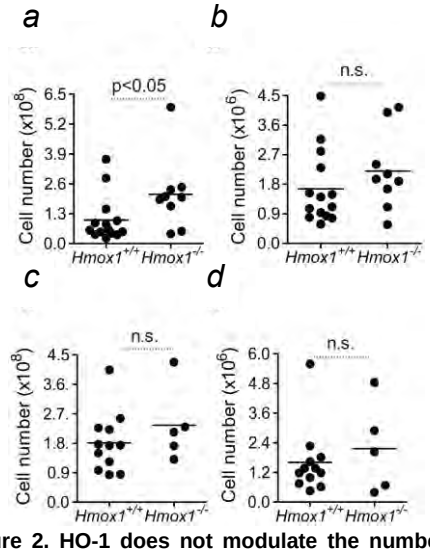

Figure 2. HO-1 does not modulate the number of cells in the CNS during EAE. Flow cytometry analysis of (***a***) splenocytes and (***b***) CNS-infiltrating cells from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=14) and *Hmox1*$^{-/-}$ (n=9) mice receiving PTx, at the peak of EAE. Data was pooled from four independent experiments. Flow cytometry analysis of (***c***) splenocytes and (***d***) CNS-infiltrating cells from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=12) and *Hmox1*$^{-/-}$ (n=5) mice receiving PTx, in the remission phase of EAE. Data was pooled from two independent experiments. Circles correspond to single mice and lines to the average of the group.

Regarding innate immune cells in the periphery, the number of DC, i.e. $CD11c^+$ cells, in the spleen of *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ mice was similar (*Fig.3a,f*) at the peak and remission phases of EAE. This was also the case for the activation status of DC from *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ mice at the peak of EAE, as revealed by the expression of MHC class II (*Fig.3b*) and of the costimulatory molecules CD40 (*Fig.3c*), CD80 (*Fig.3d*) and CD86 (*Fig.3e*). In the remission phase, the same was observed for MHC class II (*Fig.3g*), CD80 (*Fig.3i*) and CD86 (*Fig.3j*) but not for CD40 that had a modest increase in *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ DC (*Fig.3h*).

In the CNS, no differences were observed in the number of microglia cells (*Fig.4b,f*) of *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ mice at the peak or remission of EAE. The number of infiltrating-macrophages (*Fig.4a*), however, was increased and the

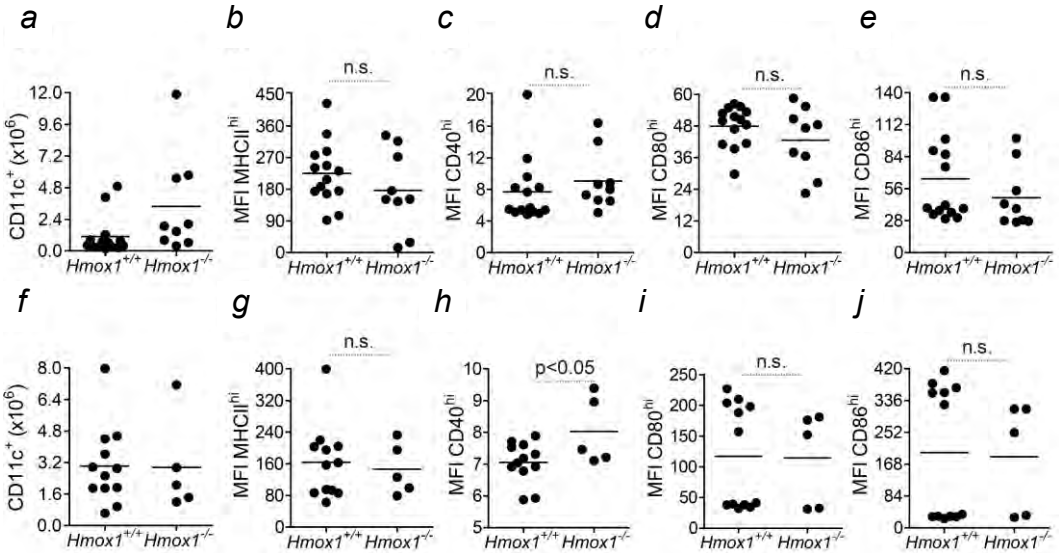

Figure 3. HO-1 does not modulate the activation status of splenic DC during EAE. Flow cytometry analysis of splenocytes from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=14) and *Hmox1*$^{-/-}$ (n=9) mice receiving PTx, at the peak of EAE. (***a***) Number of DC ($CD11c^+$). Mean intensity of fluorescence (MFI) of (***b***) MHC class II, (***c***) CD40, (***d***) CD80 and (***e***) CD86 expression in DC. Data was pooled from four independent experiments. Flow cytometry analysis of splenocytes from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=12) and *Hmox1*$^{-/-}$ (n=5) mice receiving PTx, in the remission phase of EAE. (***f***) Number of DC. MFI of (***g***) MHC class II, (***h***) CD40, (***i***) CD80 and (***j***) CD86 expression in DC. Data was pooled from two independent experiments. Circles correspond to single mice and lines to the average of the group.

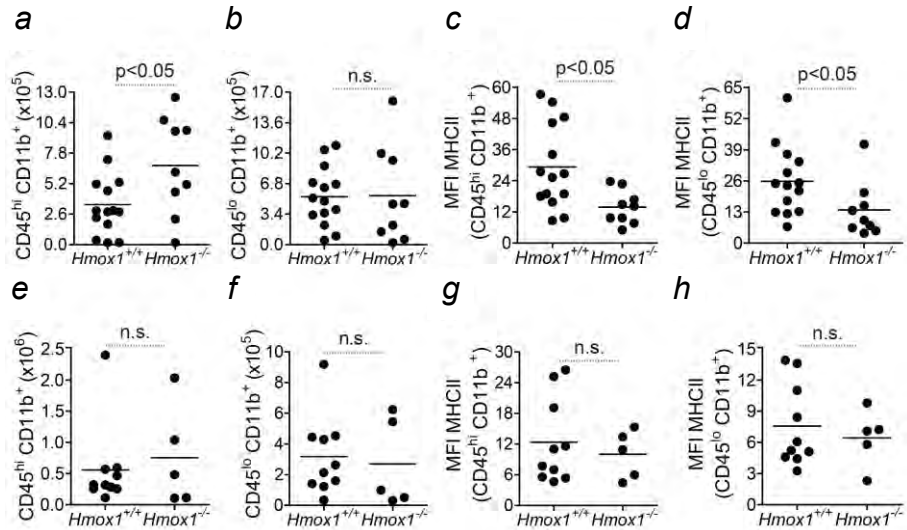

Figure 4. HO-1 does not modulate innate immune cells in the CNS during EAE. Flow cytometry analysis of CNS-infiltrating leukocytes from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=14) and *Hmox1*$^{-/-}$ (n=9) mice receiving PTx, at the peak of EAE. Number of (***a***) macrophages ($CD45^{hi}CD11b^+$) and (***b***) microglia cells ($CD45^{lo}CD11b^+$). MFI of MHC class II expression in (***c***) macrophages ($CD45^{hi}CD11b^+MHCII^{hi}$) and (***d***) microglia cells ($CD45^{lo}CD11b^+MHCII^{hi}$). Data was pooled from four independent experiments. Flow cytometry analysis of splenocytes from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=12) and *Hmox1*$^{-/-}$ (n=5) mice receiving PTx, in the remission phase of EAE. Number of (***e***) macrophages and (***f***) microglia. MFI of MHC class II expression in (***g***) macrophages and (***h***) microglia. Data was pooled from two independent experiments. Circles correspond to single mice and lines to the average of the group.

expression of MHC class II both in macrophages and microglia (*Fig.4c,d*) was reduced in *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ mice at the peak of EAE, but these differences were no longer present in the remission phase (*Fig.4e,g,h*).

No differences were observed in the number of T_H (*Fig.5a,e and 6a,e*), T_H1 (*Fig.5b,f and 6b,f*), T_H17 (*Fig.5c,g and 6c,g*) and T_{REG} (*Fig.5d,h and 6d,h*) cells in the spleen or in the CNS of *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ mice undergoing EAE. The same is true for $CD8^+$ T cells (*Fig.7a,b,e,f*), including activated $CD8^+$ T cells (*Fig.7c,d,g,h*), assessed by the production of IFN-γ.

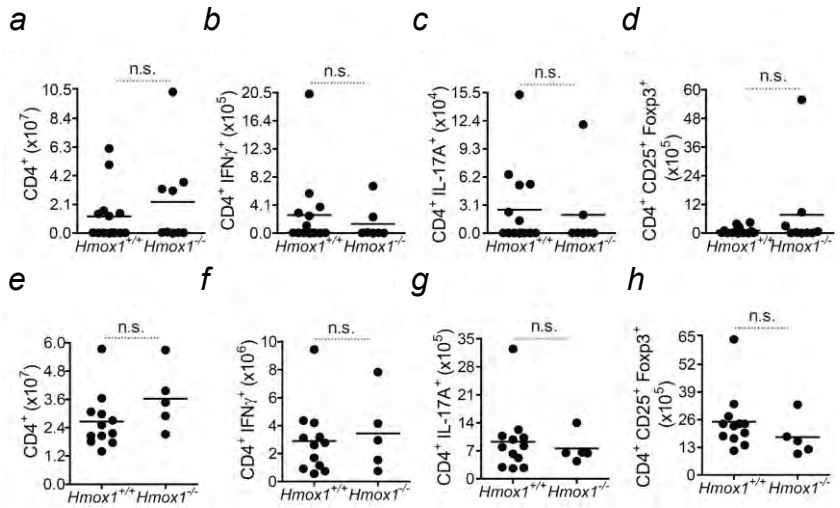

Figure 5. HO-1 does not modulate T_H and T_{REG} cell populations in the spleen during EAE. Flow cytometry analysis of splenocytes from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=14) and *Hmox1*$^{-/-}$ (n=9) mice receiving PTx, at the peak of EAE. Number of (***a***) T_H ($CD4^+$), (***b***) T_H1 ($CD4^+IFN\gamma^+$), (***c***) T_H17 ($CD4^+IL\text{-}17A^+$) and (***d***) T_{REG} ($CD4^+CD25^+Foxp3^+$). Data was pooled from four independent experiments. Flow cytometry analysis of splenocytes from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=12) and *Hmox1*$^{-/-}$ (n=5) mice receiving PTx, in the remission phase of EAE. Number of (***e***) T_H , (***f***) T_H1, (***g***) T_H17 and (***h***) T_{REG}. Data was pooled from two independent experiments. Circles correspond to single mice and lines to the average of the group.

This data suggests that *Hmox1* deletion does not affect in a significant manner innate or adaptive immune cell populations in the periphery or in the CNS during EAE in a way that could explain the increased EAE severity observed in *Hmox1*$^{-/-}$ mice compared to *Hmox1*$^{+/+}$ animals.

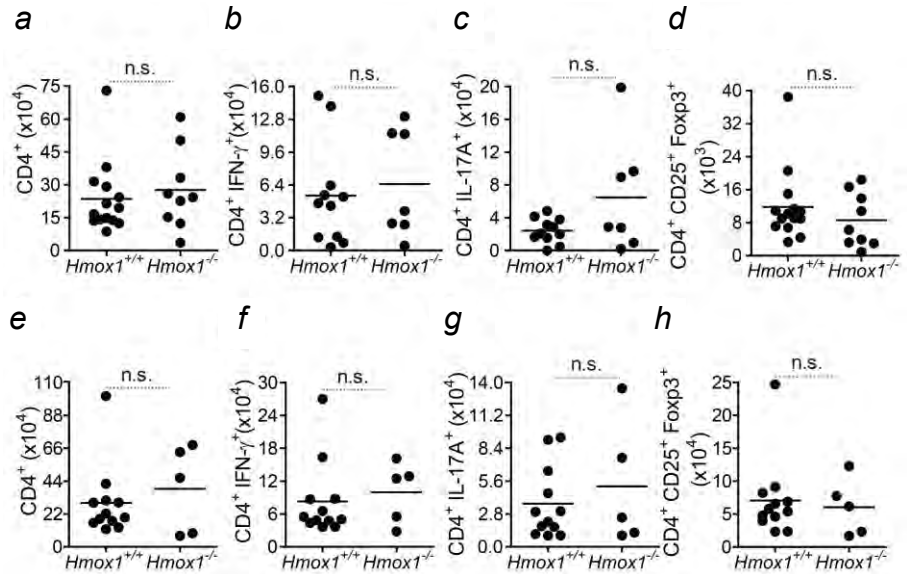

Figure 6. HO-1 does not modulate T_H and T_{REG} cell populations in the CNS during EAE. Flow cytometry analysis of CNS-infiltrating leukocytes from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=14) and *Hmox1*$^{-/-}$ (n=9) mice receiving PTx, at the peak of EAE. Number of (***a***) T_H, (***b***) T_H1, (***c***) T_H17 and (***d***) T_{REG}. Data was pooled from four independent experiments. Flow cytometry analysis of splenocytes from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=12) and *Hmox1*$^{-/-}$ (n=5) mice receiving PTx, in the remission phase of EAE. Number of (***e***) T_H, (***f***) T_H1, (***g***) T_H17 and (***h***) T_{REG}. Data was pooled from two independent experiments. Circles correspond to single mice and lines to the average of the group.

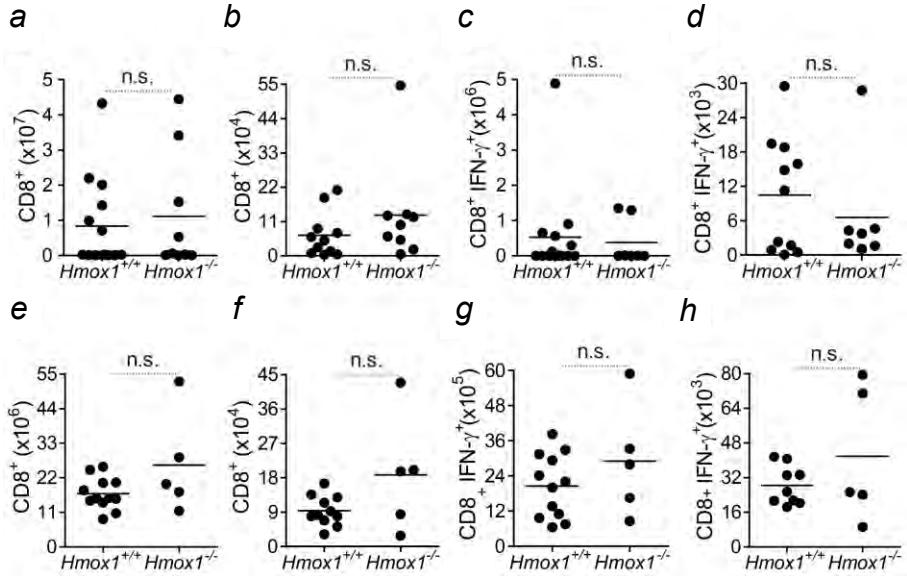

Figure 7. HO-1 does not modulate CD8$^+$ T cells in the CNS during EAE. Flow cytometry analysis of splenocytes and CNS-infiltrating leukocytes from MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=14) and *Hmox1*$^{-/-}$ (n=9) mice receiving PTx, at the peak of EAE. Number of CD8$^+$ T cells (***a***) in the spleen, (***b***) in the CNS and of activated CD8$^+$ T cells (CD8$^+$IFN-γ^+) (***c***) in the spleen and (***d***) in the CNS. Data was pooled from four independent experiments. Flow cytometry analysis of splenocytes of MOG_{35-55}-immunized *Hmox1*$^{+/+}$ (n=12) and *Hmox1*$^{-/-}$ (n=5) mice receiving PTx, in the remission phase of EAE. Data was pooled from two independent experiments. Number of CD8$^+$ T cells (***e***) in the spleen, (***f***) in the CNS and of activated CD8$^+$ T cells (***g***) in the spleen and (***h***) in the CNS. Circles correspond to single mice and lines to the average of the group.

3. Discussion

The exacerbated EAE phenotype observed in *Hmox1*$^{-/-}$ mice, compared to wild type controls (*Chapter 3 Fig.1a*), suggests that HO-1 plays a regulatory role in EAE. HO-1 is expressed during EAE in cells of the immune system, i.e. infiltrating-macrophages/microglia (*Fig1a-j*) and dendritic cells (*Chapter 3 Fig.5a*), as well as in non-hematopoietic CNS-resident cells, e.g. astrocytes (*Fig1k-m*), suggesting that its regulatory role could be exerted in the immune compartment and/or in the CNS.

Comparative analysis of innate (*Fig.3* and *4*) and adaptive (*Fig.5-7*) immune cells, in the periphery and CNS of *Hmox1*$^{-/-}$ and *Hmox1*$^{+/+}$ mice undergoing EAE did not reveal any major differences. However, *Hmox1*$^{-/-}$ mice did show a small and transient difference in specific immune cell populations, as compared to *Hmox1*$^{+/+}$ mice. However, these rather modest effects could hardly explain the strong protective effect of HO-1 expression during EAE.

These observations seem conflicting with a recent report demonstrating that specific deletion of HO-1 in myeloid cells, including macrophages and neutrophils but not DC, results in increased EAE severity due to impaired IFN-β production and thus, increased activation of macrophages and accumulation of T_H1 and T_H17 cells in the CNS[6]. Our analysis of macrophage/microglia activation (*Fig.4c,d,g,h*) and of T_H1 (*Fig5b,f and 6b,f.*) and T_H17 (*Fig.5c,g and 6c,g*) accumulation in the CNS of mice with global *Hmox1* deletion did not reveal the same. The reasons for this discrepancy are not clear at the moment. Nevertheless, it should be noted that the two animal models differ considerably in that one is deficient for HO-1 systemically while the other is a cell-specific *Hmox1*-deficient mouse. Importantly, systemic deletion of *Hmox1* results in several immune abnormalities, including splenomegaly as early as 6 weeks of age[3-5], which is not reported in mice with myeloid-specific deletion of *Hmox1*[6]. Thus, analyzing the immune response during EAE in a mouse with systemic deletion of HO-1 might not be comparable to that in a mouse with a more specific deletion, as their immune steady state is completely different.

The pharmacological induction of HO-1 at the onset of EAE decreases EAE severity (*Chapter 3 Fig.1b,c*) in a HO-1-dependent manner, since it is lost when

Hmox1$^{-/-}$ mice are used instead of *Hmox1*$^{+/+}$ (*Chapter 3 Fig.1e*). This is associated with immunoregulatory effects, i.e. reduction of MHC class II expression in APC (*Chapter 3 Fig.6a,c,d*) and down-regulation of myelin-reactive T_H cell-reactivation, proliferation (*Chapter 3 Fig.3* and *4a,e-h*) and effector cytokine production (*Chapter 3 Fig.3b* and *4b*)[7]. This data is suggestive that HO-1 could play a down-modulatory role in the immune response during EAE. Importantly, *Hmox1*$^{-/-}$ mice do not show increased expression of MHC class II in splenic DC (*Fig.3b,g*) and APC of the CNS (*Fig3b,h and 4c,d,g,h*), or increased T cell effector cytokine production (*Fig.5b,c,f,g and 6b,c,f,g and 7c,d,g,h*). This data reveals that genetic ablation of HO-1 or its pharmacological induction at the onset of disease do not have opposite immunoregulatory effects, suggesting that if the pharmacological induction approach acts specifically via HO-1 then targeting HO-1 in this manner may have therapeutic effects.

HO-1 does not modulate DC activation during EAE (*Fig.3*). This is in agreement with the observation that HO-1 also does not modulate the maturation of DC either *in vitro* (*Chapter 2 Suppl. Fig.14*) or *in vivo* (*Chapter 2 Suppl. Fig.15*) upon LPS-stimulation. Moreover, HO-1-specific deletion in DC does not seem to impact EAE severity (*Chapter 2 Fig8d-f*). This data suggests that the regulatory role of HO-1 in EAE is not mediated by expression of physiological levels of HO-1 in DC, and that the exacerbated EAE phenotype observed in mice with a myeloid-specific deletion of HO-1[6] is probably due to effects of HO-1 in other myeloid cell populations, such as macrophages and/or neutrophils.

The observation that EAE progression is similar between mice with DC-specific deletion of HO-1 and controls (*Chapter 2 Fig.8d-f*) raises the possibility that the HO-1-dependent reduction observed in EAE severity upon CoPPIX administration does not rely on a reduction of MHC class II in DC (*Chapter 3 Fig.6a*), but instead in the CNS APC, i.e. macrophages/microglia (*Chapter 3 Fig.6c,d*). It has been shown that CoPPIX-mediated reduction of MHC class II expression in DC relies on a STAT-3-dependent but HO-1-independent mechanism[8]. Nevertheless, this has been demonstrated only in GM-CSF-derived bone marrow DC, which are different from LN node DC, where the effect of CoPPIX

was analyzed, in our work. Thus, it would be important to analyze in the future: 1) MHC class II expression during EAE in *Hmox1*$^{-/-}$ *vs. Hmox1*$^{+/+}$ LN DC after CoPPIX administration and 2) MHC class II expression and the T cell response, after CoPPIX administration at the onset of EAE, in a mouse with a specific deletion of HO-1 in macrophages/microglia.

4. Methods

Mice. BALB/c *Hmox1*$^{+/-}$ mice[9] were generated by Dr. Shaw-Fang Yet (Brigham and Women's Hospital). BALB/c *Hmox1*$^{+/-}$ mice were backcrossed into the C57BL/6 background for ten generations, bred as *Hmox1*$^{+/-}$ x *Hmox1*$^{+/-}$ breeding pairs at the Instituto Gulbenkian de Ciência and genotyped as described elsewhere[7]. Food and water were provided *ad libitum*. Littermate *Hmox1*$^{+/+}$ mice were used as controls and all mice were used for experiments in the C57BL/6 background between 6 - 8 weeks of age. Mice were maintained under specific pathogen-free conditions and all experimental protocols were approved by the "Instituto Gulbenkian de Ciência animal care committee" and by the "Direcção Geral de Veterenária (DGV)" of the Portuguese Ministry of Agriculture, Rural Development and Fisheries (License 018831-2010-09-03).

Reagents. Murine MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) was obtained from Biopolymers Laboratory of Harvard Medical School (Boston, MA, USA) and dissolved in PBS (1mg/ml). Complete Freund's adjuvant (CFA) was prepared by grinding *Mycobacterium tuberculosis HR37* (4mg/ml) and re-suspending it in incomplete Freund's adjuvant (Difco; BD Biosciences). Pertussis toxin (PTx; List Biological Laboratories; Campbell, CA, USA) was dissolved in PBS (100 ng/μl). PMA (Sigma) and inonomycin (Calbiochem) were dissolved in Dimethyl sulfoxide (DMSO; 2.5 mg/ml). Brefeldin A (Epicenter technologies) was dissolved in pure ethanol (1 mg/ml).

EAE. *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ mice were immunized with MOG_{35-55} (100 μg) emulsified in CFA (100 μl) subcutaneously on each side of the belly and received

PTx intravenously (200 ng in PBS; 100 µL) after immunization (i.e. 4h and 2 days). Clinical signs of EAE were evaluated daily for 40 days and scored as follows. For classical EAE: 0 - normal; 1 - limp tail; 2 - partial paralysis of the hind limbs; 3 - complete paralysis of the hind limbs; 4 - hind-limb paralysis and forelimb weakness; 5 - moribund or deceased.

Isolation of splenocytes. Spleen cells were harvested, homogenized into a single-cell suspension in PBS 2% FCS and subjected to erythrocyte lysis in 3 ml of a hypotonic red blood cell lysis buffer (155 mM NH4Cl, 10 mM NaHCO3, 0.1 mM EDTA) while centrifuging (375g, 5 min., 4ºC). Cells were washed twice in 15 ml PBS 2% FCS (375g, 5 min., 4ºC). For intracellular cytokine staining, cells were cultured in supplemented RPMI 1640 (100 U/ml penicillin, 100 µg/ml streptomycin, 10% FCS, 50 µM β-mercaptoethanol, 10 mM HEPES and 1 mM sodium pyruvate; 2 mM L-glutamine; Invitrogen and Sigma) containing 50 ng/ml PMA, 500 ng/ml ionomycin and 10 µg/ml brefeldin A for 4h at 37°C (5% CO_2, 95% humidity). Surface and intracellular stainings were analyzed by flow cytometry.

CNS-infiltrating leukocytes. Brain and spinal cord were harvested from transcardially perfused mice (20 ml cold PBS). Single-cell suspensions were prepared in HBSS (Invitrogen) containing Collagenase VIII (0.2 mg/ml; Sigma) by homogenizing the tissue between two glass slides, digesting (30 min.; 37°C; 5% CO_2, 95% humidity) and filtering (100-µm; BD Falcon). After centrifugation (375g, 5 min., 4ºC) in PBS, leukocytes were separated on a discontinuous 30% percoll gradient (Sigma) by centrifugation (1041g, 30 min., RT) erythrocytes were lysed in hypotonic red blood cell lysis buffer (5 min., RT). For intracellular cytokine staining, cells were cultured in supplemented RPMI 1640 with PMA, ionomycin and brefeldin A, as described above. Surface and intracellular stainings were analyzed by flow cytometry.

Flow cytometry and antibodies. The following mAb were used: FcγIII/II receptor (2.4G2), anti-CD4 (RM4-5), anti-CD11b (M1/70), anti-CD25 (PC 61), anti-CD11c (HL3), anti-CD40 (3/23), anti-CD45 (30-F11), anti-CD80 (16-10A1), anti-CD86

(GL1) (prepared *in house* from hybridoma culture supernatants), anti-CD8 (YTS169.4; BD Biosciences) and anti-MHC class II (AF6-120.1; BD Biosciences and M5/114; eBioscience). Antibodies were directly conjugated to FITC, Alexa488, PE, PerCP, APC, Cy5 or Alexa467.

For surface staining, cells were incubated with an anti-FcγIII/II receptor antibody (2.4G2) in PBS 2% FCS (20 min., 4°C). Surface staining was performed in PBS 2% FCS (20 min., 4°C). After washing and centrifugation (666g, 2 min., 4ºC), cells were incubated with streptavidin–allophycocyanin for (20 min., 4°C) to detect biotinylated antibodies. For flow cytometry analysis of surface stainings dead cells were excluded using propidium iodide (1.7 μg/ml).

For intracellular cytokine staining cells were fixed (2% paraformaldehyde; 30 min., RT) and permeabilized (0.5% saponin in PBS 2% FCS, i.e. permeabilization buffer)(10 min., RT). Cells were incubated with anti-IFN-γ (XMG1.2; BD Biosciences) or anti-IL-17 (eBioTC11-18H10.1; eBioscience) mAb in permeabilization buffer (20 min., 4°C).

For intracellular Foxp3 staining, cells were stained with anti-mouse Foxp3 conjugated (FJK-16s; eBioscience) with PE using the Foxp3 detection set (eBioscience) according to the manufacturer's instructions.

Cell numbers were assessed by flow cytometry, using a fixed number of latex beads (Beckman Coulter) co-acquired with a pre-established volume of the cellular suspensions.

Flow cytometry detection was done in the FACSCalibur (BD Biosciences), using the cellquest software (BD Biosciences) for acquisition. Post-acquisition analysis was performed with FloJo software (Treestar).

Histology. Mice were perfused with PBS (20 ml) followed by 4% paraformaldehyde (PFA) (50 ml) and post-fixed overnight in PFA 4%. Brain and spinal cord were extracted from the bone with forceps and spinal cord embedded in 3% agar. Sections were prepared in a vibratome (30 μm) and hematoxilin-eosin staining performed as follows. Briefly, sections were placed in gelatin-coated slides and immersed in a hematoxilyn solution for ten minutes, washed with water and

immersed in a solution of eosin for 15 minutes. Following, sections were washed with water and dehydrated in a bath of alcohols for two minutes in each, according to the following order 70% (once), 96% (twice) 100% (twice) ethanol and Xilol.

Immunofluorescence of the CNS. CNS sections were prepared as described above and the following antibodies were used: rabbit anti-HO-1 polyclonal (SPA895; Stressgene Biotechnologies), rat anti-CD11b monoclonal (Leinco), rat anti-mouse F4/80 monoclonal (Serotec) and rat anti-GFAP monoclonal (Calbiochem) antibodies. Secondary antibodies directly coupled to fluorochromes used were a goat anti-rabbit Alexa 568 (Invitrogen) to detect HO-1, and an anti-rat Alexa 488 (Invitrogen) to detect CD11b, F4/80 and GFAP. Briefly, sections were blocked with 10 % normal goat serum (NGS) (Sigma) with 0.5% Triton-X (Sigma) in PBS for 10 min., washed with PBS and incubated with the primary antibody diluted in 1% NGS with 0,5% Triton-X in PBS for 90 min. at RT or ON at 4ºC. Sections were washed again in PBS and incubated with the secondary antibody diluted in 1% NGS with 0,5% Triton-X in PBS for 90 min. at RT in the dark. At the end of this time, sections were washed with PBS, placed in a gelatin-coated slide and mounted in the water soluble mounting medium Mowiol (Sigma) with a coverslip on top. After 30 min. at 4ºC sections were analysed by fluorescence microscopy.

Statistics. Statistical analysis was performed using the Nonparametric Mann-Whitney *U* test, when sample size was smaller than 5, or did not follow normal distribution, and when it did Unpaired Student's t-test for unequal variances was used. Normal distributions were confirmed using the Kolmogorov-Smirnov test. $p < 0.05$ was considered statistically significant.

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Appendix 2

Immunoregulatory effects of HO-1: how does it work?

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Abstract

The heme-catabolizing enzyme heme oxygenase-1 (HO-1; encoded by the *Hmox1* gene) inhibits the pathogenesis of several immune-mediated inflammatory diseases. This unusually broad salutary effect is thought to rely on the immunoregulatory actions of HO-1, exerted on innate and adaptive immune cells. According to this notion, HO-1 'dampens' innate and adaptive immune responses, limiting immune-mediated tissue injury and thus suppressing the pathogenesis of immune-mediated inflammatory diseases. We will argue that the salutary effects of HO-1 are also exerted via its cytoprotective action, which sustains tissue function and prevents unfettered immune activation by endogenous pro-inflammatory ligands released from injured cells.

The heme-catabolizing enzyme heme oxygenase-1 (HO-1)

HO-1 is a ubiquitously expressed stress-responsive enzyme that catabolizes iron (Fe) protoporphyrin IX (i.e. heme) into equimolar amounts of labile Fe, biliverdin and carbon monoxide (CO)[1] (*Fig.1*). In contrast to the Fe contained within the protoporphyrin IX ring of heme, which can catalyze the production of free radicals via the Fenton chemistry, several Fe metabolic pathways can neutralize the labile Fe produced through heme catabolism by HO-1. Among those, the induction of ferritin heavy chain (FtH) expression by labile Fe mediates to a large extent the protective effects of HO-1[2] (*Fig.1*). The biliverdin produced through heme catabolism by HO-1 can be converted, by biliverdin reductase, into the cytoprotective antioxidant bilirubin[3]. Moreover, CO is also cytoprotective[4] as well as anti-inflammatory[5] (reviewed in[6]). Presumably, these end-products of heme catabolism can each alone or in combination contribute to the protective effects of HO-1 (reviewed in[7,8]). In addition, heme degradation should be protective per se, as it prevents the deleterious (pro-oxidant) effects of free heme.

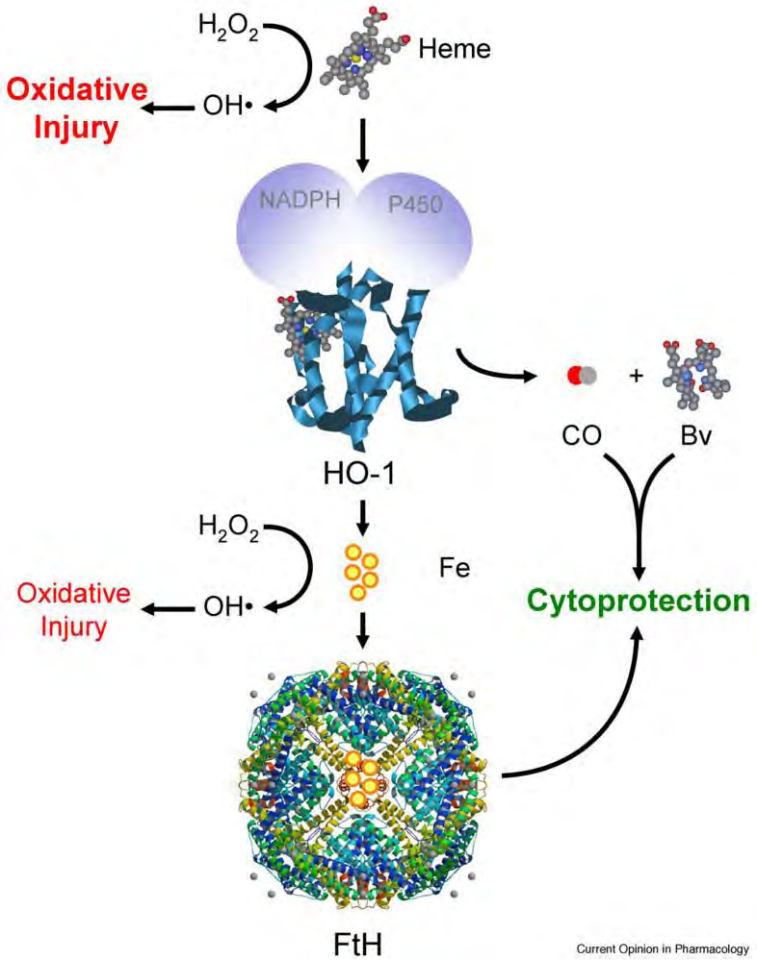

Figure 1. The heme/HO-1 system. Free heme is highly pro-oxidant, as its Fe atom can participate in the Fenton chemistry via which hydrogen peroxide (H_2O_2) is converted into the highly reactive hydroxyl radical ($OH^{\bullet}$). This pro-oxidant effect is controlled by several mechanisms including heme catabolism by HO-1 (Protein Data Base; PDB 1n3u), a reaction assisted by NADPH and Cytochrome P450. Contrary to heme-Fe, labile Fe (yellow circles) can be neutralized by FtH (Protein Data Base; PDB 1r03). The salutary (cytoprotective) effects of HO-1 are mediated to a large extent by this conversion of reactive heme-Fe (that promotes oxidative injury) into chelatable labile Fe^{2}. Bv: Biliverdin.

Immunoregulatory effects of HO-1

The notion that HO-1 is immunoregulatory is supported by the observation that *Hmox1* deficiency leads, in mice and human(s), to the spontaneous development of a chronic inflammatory pathology characterized by increased blood leukocyte count, serum IgM, accumulation of polymorphonuclear (PMN) cells, and

monocyte/macrophages (Mø) in the spleen as well as in nonlymphoid tissues and widespread oxidative tissue injury[9-11]. We will review hereby data supporting the notion that the immunoregulatory effects of HO-1 are exerted via its expression on innate and adaptive immune cells, but perhaps more importantly, by its expression in nonlymphoid tissues where it affords cytoprotection against oxidative injury.

Effects of the heme/HO-1 system on innate immunity

Under homeostasis, heme exists essentially as a prosthetic group in several hemoproteins. However, under pathologic conditions noncovalently bound heme can be released from those hemoproteins[12] (reviewed in[13]). The free heme produced in this manner is recognized by the pattern recognition receptor (PRR) toll-like receptor 4 (TLR4), triggering the production of low levels of pro-inflammatory cytokines by innate immune cells such as Mø[14] (*Fig.2*). However, when pre-exposed to free heme, Mø become 'desensitized' that is lose the ability to respond, to a subsequent challenge by other TLR4 agonists, for example bacterial lipopolysaccharide (LPS) (*Fig.2*)[8]. This immunoregulatory effect is credited to the induction of HO-1 and in particular to the production of CO[5]. Interestingly, the anti-inflammatory effect of other immunoregulatory molecules such as IL-10[15] and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2)[16] also appears to be exerted via this mechanism, namely the induction of HO-1 in Mø (reviewed in[17]). While suggestive that HO-1 can exert anti-inflammatory effects when expressed in Mø, these studies have not demonstrated that those effects occur under physiologic conditions. There is evidence however, to suggest that this might not be the case. For instance, peritoneal Mø isolated from *Hmox1*$^{-/-}$ or *Hmox1*$^{+/+}$ mice have a similar pro-inflammatory response to LPS *in vitro*[9], making it unclear whether HO-1 exerts immunoregulatory effects when expressed physiologically in Mø.

Similar to Mø, PMN cells can 'sense' free heme, in this case through a yet unidentified G-protein-coupled receptor[18,19]. As such, free heme can act as a PMN cell chemoattractant, activating PMN cells to produce reactive oxygen species as well as pro-inflammatory cytokines[18,19] (*Fig.2*). Since free heme can act as a potent

pro-oxidant catalyst via the Fenton chemistry, this latter effect might exacerbate Mø as well as PMN-driven oxidative tissue injury. Therefore, by limiting the availability of free heme, HO-1 might inhibit PMN cell chemotaxis and activation, and thus, oxidative tissue injury. This notion is supported by the observation that *Hmox1* deficiency, in mice and humans, is associated with widespread PMN cell activation and tissue infiltration, as well as with oxidative tissue injury[9-11,20]. In addition, pharmacologic induction of HO-1 inhibits the activity of the $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ subunits of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, thereby reducing the production of reactive oxygen species by activated PMN cells and Mø[21,22], contributing further to limit oxidative tissue injury.

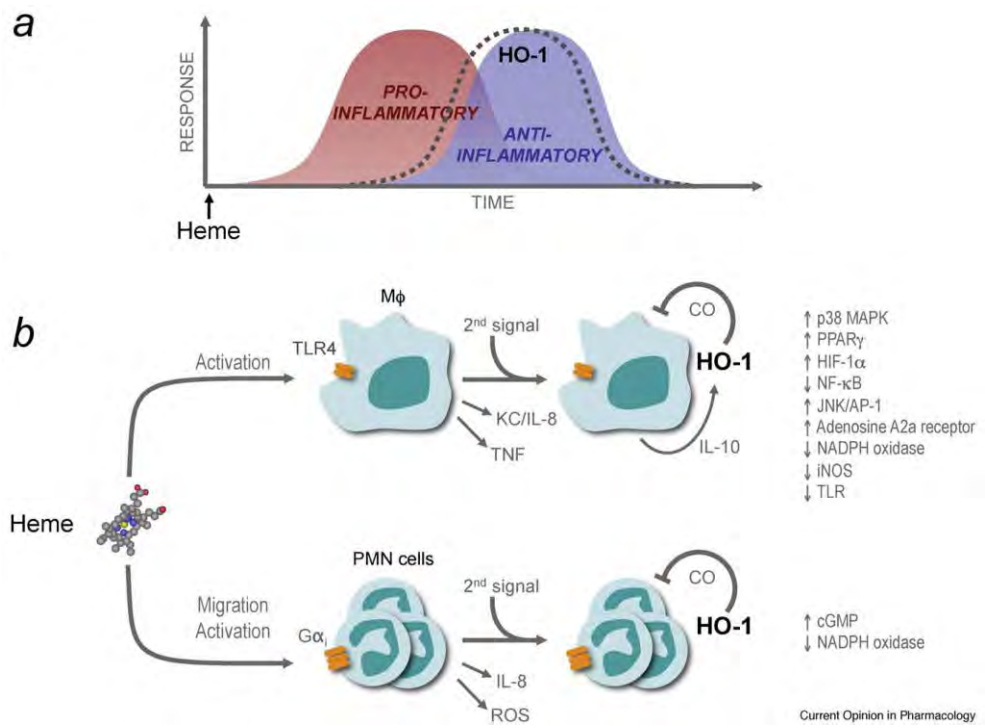

Figure 2. Regulation of innate immunity by the heme/HO-1 system. (***a***) Free heme induces a mild pro-inflammatory response followed by a regulatory response attributable to the induction of HO-1. (***b***) Free heme can be recognized by mouse Mø, via TLR4, which triggers the production of keratinocyte chemokine (KC/IL-8) and TNF[14]. In addition, free heme induces the expression of HO-1 in Mø, which exerts a series of anti-inflammatory effects against a variety of pro-inflammatory agonists (second signal). These effects are mediated via the regulation of several signal transduction pathways by CO (listed to the right in gray). Free heme is recognized by PMN cells, via an unidentified G-protein-coupled receptor that triggers PMN cell migration as well as the production of IL-8 and reactive oxygen species[19]. Heme-driven HO-1 induction inhibits PMN cell activation by reducing the availability of free heme and by inhibiting NADPH activity, an effect mediated by CO.

Dendritic cells (DC) 'bridge' innate and adaptive immune responses, migrating upon activation, from tissues into secondary lymphoid organs, as to become immunogenic, that is acquire the ability to activate naïve T cells. Mouse immature DC express negligible levels of HO-1, inducing its expression upon activation[23,24]. Intriguingly, rat and human immature DC express HO-1 constitutively, downregulating its expression upon activation[25]. In any case, pharmacologic induction of HO-1 inhibits mouse, rat, and human DC activation and immunogenicity[25-27] (*Fig.3a*), an effect mimicked by pharmacologic delivery of CO[26] or biliverdin/bilirubin[28] (*Fig.3a*). This suggests that CO and/or biliverdin/bilirubin mediate the immunosuppressive effects associated with pharmacologic modulation of HO-1 expression in DC (*Fig.3a*). However, the physiological relevance of these observations is somehow questioned by the recent observation that the pharmacologic modulators of HO-1 used in these studies can exert immunosuppressive effects in DC, independently of HO-1[23]. Moreover, the observation that activation of DC from *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mice occurs in a similar manner *in vitro*[23] questions further the physiological relevance attributed to the expression of HO-1 in DC. As discussed below, HO-1 might exert immunoregulatory effects on innate immune cells, including DC, via a mechanism that does not rely on its expression in these cells.

Effects of the heme/HO-1 system on adaptive (T cell) immunity

Several observations *in vitro* suggest that HO-1 exerts immunoregulatory effects by inhibiting T cell activation, proliferation and/or effector function. First, the activation of human CD4$^+$ T helper (T_H) cells is associated with a significant induction of HO-1 expression[29] (*Fig.3b*). Second, pharmacologic induction of HO-1 inhibits human T_H and CD8$^+$ cytotoxic T (T_C) cell activation[29]. Third, CO inhibits T_H cell activation[29] and induces apoptosis in Jurkat T cells[30] (*Fig.3b*). Fourth, biliverdin/bilirubin inhibits mouse and human T_H cell activation[28] (*Fig.3b*). In keeping with these observations, pharmacologic induction of HO-1 *in vivo* can drive activated T_H cells to undergo apoptosis via activation induced cell death[31] (*Fig.3b*) and can promote dominant

peripheral T cell tolerance against transplanted organs[32]. This latter effect is mimicked by biliverdin/bilirubin[28,33], which suppresses T cell-driven inflammatory pathologies such as the rejection of transplanted organs[33] or autoimmune neuroinflammation[28]. The immunoregulatory effects of biliverdin/bilirubin are mediated via inhibition of the transcription factors, nuclear factor of activated T-cells (NF-AT) and nuclear factor kappa B (NF-κB), which suppresses IL-2 production in T_H cells[28,33]. Of notice, both biliverdin and bilirubin are endogenous ligands for the aryl hydrocarbon receptor (AHR)[34], which regulates the plasticity of T_H cell phenotype toward an anti-inflammatory regulatory T (T_{REG}) cell or a pro-inflammatory T_H17 effector function[35,36]. Although speculative, it is possible that the immunoregulatory effects of HO-1 might be exerted, to at least some extent, through the production of these endogenous AHR ligands.

HO-1 has been suggested to exert immunoregulatory effects by modulating T_{REG} cell function[37] (*Fig.3c*), a notion supported by the following set of observations. First, human and mouse T_{REG} cells express HO-1 constitutively[38,39] (*Fig.3c*). Second, pharmacologic inhibition of HO-1 suppresses human T_{REG} cell function *in vitro*[40]. Third, the activity of *Hmox1*$^{+/+}$ T_{REG} cells is compromised *in vitro* by *Hmox1*$^{-/-}$ DC[41], a finding we have not been able to reproduce[39]. While these observations suggest that HO-1 regulates T_{REG} cell function *in vitro*, our finding that T_{REG} cell development as well as peripheral maintenance and function are normal in *Hmox1*$^{-/-}$ mice[39], questions the physiologic relevance of the effects attributed to pharmacologic modulation of HO-1 in T_{REG} cells.

Of interest, free heme can act as a T cell mitogen *in vitro*[42-44], promoting T_H cell activation, in a major histocompatibility complex (MHC) class II restricted manner[43]. Free heme also promotes the activation and effector function of T_C cells *in vitro*, but in this case via a mechanism that is not completely MHC class I restricted[44]. Although the molecular basis for heme recognition by T cells and its putative impact on the outcome of immune-mediated inflammatory diseases remain unexplored, these observations suggest that HO-1 might regulate T cell activation by decreasing free heme availability.

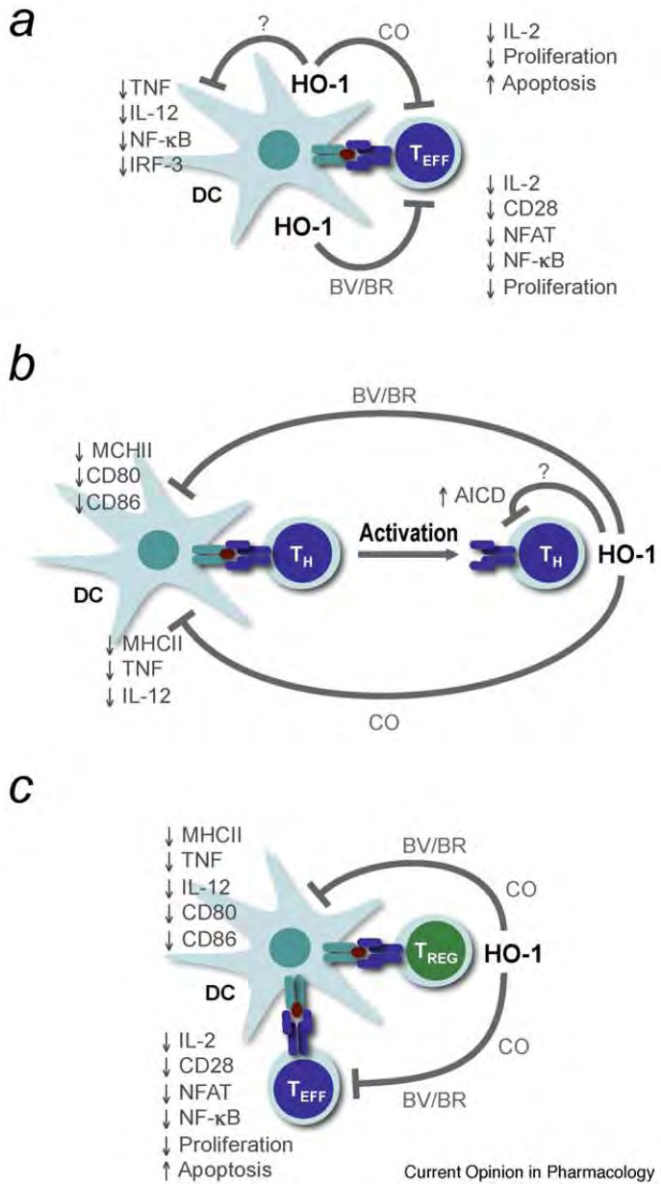

Figure 3. Regulation of adaptive immunity by the heme/HO-1 system. Immunoregulatory effects attributed to HO-1 expression in (***a***) DC, (***b***) activated T_H cells, and (***c***) T_{REG} cells. In all three models (panels a, b and c) HO-1 exerts immunoregulatory effects via the paracrine action of the end-products of heme catabolism, namely CO, BV/BR, or by a yet unidentified mechanism associated with pharmacologic induction of HO-1. AICD: activation induced cell death, BV/BR: biliverdin/bilirubin, T_{EFF}: effector T cell (T_H or T_C).

Cytoprotective effects of HO-1

Many if not all immune-mediated inflammatory diseases are associated with the production of free radicals, leading to some level of cellular oxidative stress and oxidative tissue injury. If not controlled, oxidative stress can drive cells to undergo necrosis, a form of cell death associated with the release of their intracellular content, including uric acid[45] and high mobility group box 1 (HMGB1)[46] (*Fig.4*). These intracellular components can be recognized by PRR expressed in innate immune cells, such as Mø and DC, and thus can act as pro-inflammatory and eventually immunogenic agonists (reviewed in[47]). Given the above, mechanisms regulating cell death/survival in tissues should exert immunoregulatory effects that impact on the outcome of immune-mediated inflammatory diseases[48]. The cytoprotective effect of HO-1might act in such a manner (*Fig.4*).

When challenged by a pro-inflammatory agonist such as LPS, *Hmox1*$^{-/-}$ mice succumb to unfettered oxidative stress[9,20], associated with widespread oxidative tissue injury and end-stage multi-organ failure[9,49]. This suggests that HO-1 prevents the pathologic outcome of inflammatory responses by affording cytoprotection against oxidative stress. In subsequent studies, the cytoprotective effect of HO-1 was associated with protection against many other immune-mediated inflammatory diseases[50] (reviewed in[7]).

Expression of HO-1 or exposure to the by-products of its enzymatic activity is cytoprotective against oxidative injury in a wide variety of cells, including fibroblasts, vascular endothelial cells, pancreatic β-cells, hepatocytes, kidney epithelial cells, cardiac myocytes and central nervous system astrocytes and neurons, among others (reviewed in[7,8,51]). This broad cytoprotective effect might account for the equally broad protective effects of HO-1 against the development of vascular diseases, diabetes, liver dysfunction, kidney failure, myocardial infarction as well as diseases of the central nervous system (reviewed in[7]).

Presumably, the cytoprotective effect of HO-1 has a dual salutary role, in that it sustains tissue/organ function while inhibiting the release of endogenous pro-inflammatory ligands from injured cells (*Fig.4*). This latter effect should contribute in

a significant manner to prevent unfettered inflammation. In keeping with this notion, *Hmox1*$^{-/-}$ mice produce high levels of circulating endogenous pro-inflammatory ligands, for example HMGB1, in response to LPS[52], an effect that promotes the development of septic shock[52,53]. Moreover, pharmacologic induction of HO-1 affords tissue cytoprotection and inhibits the production of extracellular HMGB1 in response to LPS[54]. While expression of HO-1 might inhibit the release of other endogenous pro-inflammatory ligands this has not been tested critically.

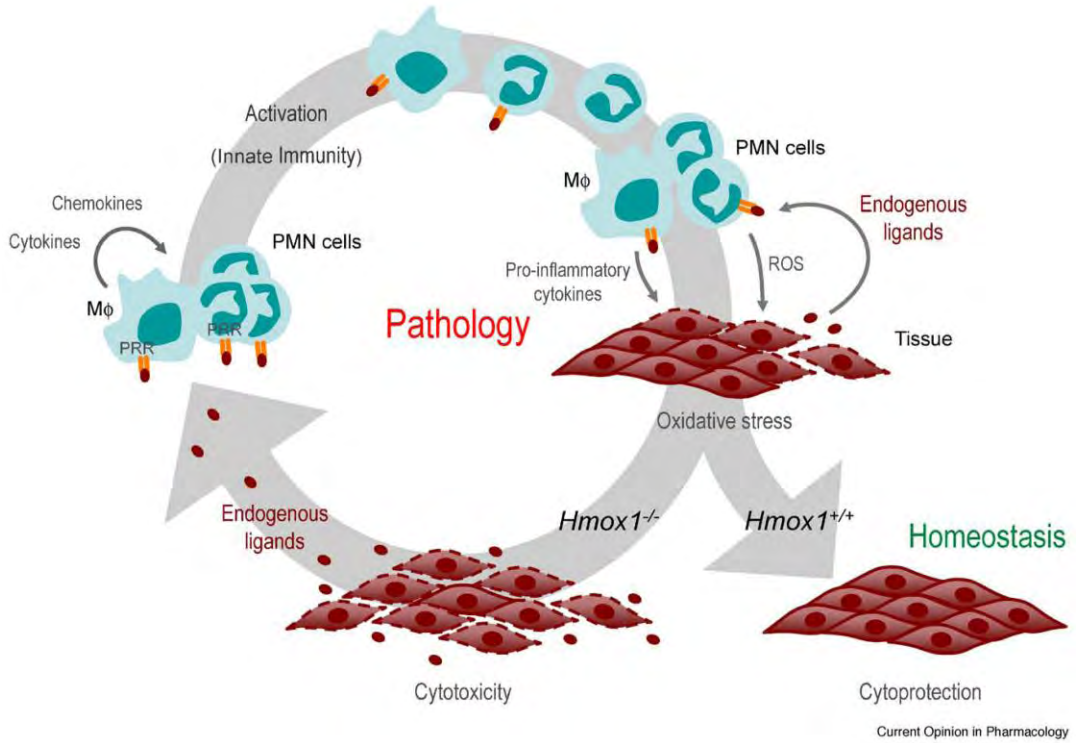

Figure 4. Cytoprotective effect of the heme/HO-1 system. Activation of innate immune cells, including Mø and PMN cells is associated with the production of ROS and pro-inflammatory cytokines, leading to oxidative stress. Expression of HO-1 in response to oxidative stress (i.e. *Hmox1*$^{+/+}$) is cytoprotective and prevents the release of endogenous pro-inflammatory ligands from injured cells, thus promoting the resolution of inflammation and the return to homeostasis. When expression of HO-1 is impaired (i.e. *Hmox1*$^{-/-}$) oxidative stress leads to widespread programmed cell death (cytotoxicity) resulting in impaired tissue function as well as release of endogenous pro-inflammatory ligands. By engaging PRR in innate immune cells, endogenous ligands sustain the activation of innate immune cells (inflammation) and exacerbate tissue injury, an effect that should promote the development of immune-mediated inflammatory diseases.

Assuming that tissue cytoprotection contributes to the salutary effects of HO-1, other cytoprotective genes should also prevent the pathogenesis and/or progression of immune-mediated inflammatory diseases. This is likely to be the

case for at least another ubiquitously expressed stress-responsive cytoprotective gene, namely *Fth* (*Fig.1*). The mechanism underlying the salutary effect of HO-1 and presumably that of FtH against immune-mediated inflammatory diseases is not clear. We speculate that this might be related to our recent finding that free heme can sensitize cells in non-hematopoietic tissues to undergo programmed cell death, a deleterious effect that should promote the pathogenesis of a number of immune-mediated inflammatory diseases (E Seixas *et al.*, *unpublished observation*). Expression of HO-1 (E Seixas *et al.*, *unpublished observation*) as well as FtH (R Gozzellino *et al.*, *unpublished observation*) affords cytoprotection against heme-driven programmed cell death, which should inhibit the pathogenesis of immune-mediated inflammatory diseases.

Concluding remarks

It has been assumed that the broad protective effects of HO-1 are mediated essentially through its immunoregulatory effects exerted in cells of the innate (i.e. Mø, PMN cells, and DC) or adaptive (i.e. T cells) immune system. We have argued hereby that the immunoregulatory effects of HO-1 are exerted to a large extent via its cytoprotective effect in tissues, which should have important implications for our current understanding of not only the mechanisms of action of HO-1 but also of those underlying the pathogenesis of a variety of immune-mediated inflammatory diseases.

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